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170. THE EFFECT OF COMMERCIAL STERILIZATION ON THE NUTRITIVE VALUE OF MILK

BY S. K. KON AND K. M. HENRY
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WITH THE COLLABORATION OF

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I. INTRODUCTION

By S. K. KON and K. M. HENRY

WE have dealt in a previous communication (1) with the effects of commercial pasteurization on the nutritive value of milk. We present here the results of an enquiry into the nutritional effects of the more drastic heat treatment to which milk is subjected during commercial sterilization. There is no legal definition of either sterilized milk or of the process of sterilization, but in commerce it is customary to apply the term to milk which has been heated to at least 212° F. for varying lengths of time.

Large quantities of such milk are consumed in certain urban areas in this country, especially in London and in the Midlands. The practice is spreading also to other large towns where sterilized milk is being increasingly used in infant feeding (2). An investigation of the nutritional value of sterilized milk is therefore of definite practical importance. In planning the experiments and methods we followed on the whole our earlier work on pasteurized milk (1). As before, the heated and raw milks were from the same bulk and the sterilization was carried out commercially by a method used (with minor alterations) for the bulk of the sterilized milk produced in this country.

EXPERIMENTAL

The method of treatment of the raw and sterilized milks

The raw and sterilized milks were both obtained from a large processing depot, the procedure of sterilization being (according to information supplied) as follows:

Raw milk was collected once or twice a day according to the season. Upon receipt at the dairy it was immediately cooled to 40° F. and was transported in insulated glass-enamelled tanks to the Processing Depot. It was then heated to 160° F. by flash heating and homogenized at about 2 tons pressure. The hot homogenized milk was then put into open bottles which were placed in an

Table I. *Temperature of raw milk on receipt in the laboratory, percentages of fat and total solids in and bacteriological tests on the experimental milks*

Date	Temp. ° F. of raw milk when received	% fat		% total solids		Bacteriological tests			
						Raw		Sterilized	
		Raw	Sterilized	Raw	Sterilized	Count/ml.*	Coliform organisms†	Count/ml.‡	Coliform organisms†
21. ix. 36	52	3·60	3·55	12·27	12·26	15,000,000	+ + + + + −	Nil	− − − − − −
22. ix. 36	50	3·60	3·60	12·24	12·21	6,680,000	+ + + + + −	Nil	− − − − − −
23. ix. 36	50	4·05	4·05	12·95	12·90	Millions	+ + + + + −	20	− − − − − −
24. ix. 36	51	3·65	3·65	12·37	12·36	8,000,000	+ + + + + −	Nil	− − − − − −
25. ix. 36	58	4·15	4·15	12·83	12·85	Millions	+ + + + + +	100	− − − − − −
26. ix. 36	58	4·15	4·15	12·83	12·85	—	—	—	—
27. ix. 36	58	4·15	4·15	12·83	12·85	—	—	—	—
28. ix. 36	48	4·05	4·05	12·90	12·90	—	—	—	—
29. ix. 36	47	4·05	4·05	12·99	12·98	12,800,000	+ + + + + −	Nil	− − − − − −
30. ix. 36	49	4·15	4·10	13·02	12·98	1,070,000	+ + + + + −	Not done	
1. x. 36	52	4·05	4·05	13·04	12·99	4,500,000	+ + + + + −	Nil	− − − − − −
2. x. 36	55	3·90	3·90	12·92	12·88	—	—	—	—
3. x. 36	55	3·90	3·90	12·92	12·76	—	—	—	—
4. x. 36	51	3·80	3·80	12·73	12·76	—	—	—	—
5. x. 36	47	3·90	3·85	12·84	12·80	—	—	—	—
6. x. 36	52	3·85	3·80	12·79	12·75	3,400,000	+ + + + + −	Nil	− − − − − −
7. x. 36	47	3·95	3·95	12·81	12·81	700,000	+ + + − − −	Nil	− − − − − −
8. x. 36	45	3·80	3·80	12·79	12·72	—	—	—	—
9. x. 36	51	3·80	3·80	12·78	12·75	—	—	—	—
10. x. 36	51	3·80	3·80	12·78	12·75	—	—	—	—
11. x. 36	49	3·75	3·75	12·70	12·76	—	—	—	—
12. x. 36	50	3·75	3·75	12·72	12·78	—	—	—	—
13. x. 36	46	3·70	3·65	12·50	12·51	—	—	—	—
14. x. 36	49	3·85	3·85	12·75	12·69	2,500,000	+ + + + + + −	Nil	− − − − − −
15. x. 36	47	3·65	3·75	12·59	12·68	—	—	—	—
16. x. 36	54	3·80	3·75	12·74	12·71	34,800,000	+ + + + + + −	Nil	− − − − − −
17. x. 36	54	3·80	3·75	12·74	12·71	—	—	—	—
18. x. 36	54	3·60	3·55	12·54	12·71	—	—	—	—
19. x. 36	50	3·90	3·85	12·77	12·69	—	—	—	—
20. x. 36	50	3·85	3·80	12·75	12·70	1,160,000	+ + + + + + −	Nil	− − − − − −
21. x. 36	46	4·10	4·05	12·95	12·86	—	—	—	—
22. x. 36	49	3·90	3·90	12·75	12·75	2,100,000	+ + + + − −	Nil	− − − − − −
23. x. 36	54	3·80	3·75	12·71	12·69	—	—	—	—
24. x. 36	54	3·80	3·75	12·71	12·69	—	—	—	—
25. x. 36	48	3·80	3·80	12·55	12·60	—	—	—	—
26. x. 36	52	3·85	3·85	12·62	12·74	—	—	—	—
27. x. 36	51	3·95	4·00	12·80	12·77	26,800,000	+ + + + + +	54	+ − − − − − §
28. x. 36	49	3·95	3·85	12·90	12·75	460,000	+ − − − − −	Nil	− − − − − −
29. x. 36	48	4·05	4·05	12·91	12·89	2,400,000	+ + + + − −	1000	− − − − − −
30. x. 36	55	3·90	3·95	12·70	12·83	—	—	—	—
31. x. 36	58	3·90	3·95	12·70	12·83	—	—	—	—
1. xi. 36	53	3·95	4·05	12·75	12·78	31,200,000	+ + + + − −	20	− − − − − −
2. xi. 36	49	4·00	3·95	12·89	12·80	—	—	—	—
3. xi. 36	47	3·95	3·95	12·70	12·72	650,000	+ + + + − −	Nil	− − − − − −
4. xi. 36	47	4·20	4·20	13·10	13·06	7,000,000	+ + + + − −	Nil	− − − − − −
5. xi. 36	48	4·10	4·10	12·88	12·95	—	—	—	—
6. xi. 36	50	4·05	4·05	12·94	12·90	—	—	—	—
7. xi. 36	50	4·05	4·05	12·94	12·90	—	—	—	—
8. xi. 36	44	4·00	4·05	12·78	12·79	—	—	—	—
9. xi. 36	49	4·10	4·15	12·92	12·92	—	—	—	—

Table I (*continued*)

Date	Temp. °F. of raw milk when received	% fat		% total solids		Bacteriological tests			
						Raw		Sterilized	
		Raw	Sterilized	Raw	Sterilized	Count/ml.*	Coliform organisms†	Count/ml.‡	Coliform organisms†
10. xi. 36	45	3·90	3·95	12·80	12·76	—	—	—	—
11. xi. 36	46	3·95	3·95	12·86	12·79	1,240,000	+ + + + – –	Nil	– – – – – –
12. xi. 36	50	4·00	4·00	12·77	12·83	7,300,000	+ + + + – –	Nil	– – – – – –
13. xi. 36	46	3·95	4·00	12·75	12·82	—	—	—	—
14. xi. 36	46	3·95	4·00	12·75	12·82	—	—	—	—
15. xi. 36	47	4·00	4·05	12·73	12·73	—	—	—	—
16. xi. 36	49	4·00	4·00	12·83	12·79	4,600,000	+ + + + – –	Nil	– – – – – –
17. xi. 36	55	4·00	4·00	12·71	12·71	16,600,000	+ + + + + +	Nil	– – – – – –
18. xi. 36	48	3·90	3·85	12·53	12·55	37,700,000	+ + + + + –	70	– – – – – –
19. xi. 36	50	3·95	4·05	12·83	12·82	13,700,000	+ + + + + –	40	– – – – – –
20. xi. 36	47	3·95	3·95	12·92	12·93	30,000,000	+ + + + – –	50	– – – – – –
21. xi. 36	47	3·95	3·95	12·92	12·93	—	—	—	—
22. xi. 36	47	4·05	3·95	12·87	12·93	—	—	—	—
23. xi. 36	45	3·95	3·95	12·63	12·61	600,000	+ + + – – –	50	– – – – – –
24. xi. 36	45	3·85	3·80	12·87	12·81	—	—	—	—
25. xi. 36	43	4·00	4·05	13·00	13·04	—	—	—	—
26. xi. 36	45	4·20	4·20	13·11	13·16	250,000	+ + + + – –	10	– – – – – –
27. xi. 36	46	3·85	3·85	12·89	12·86	—	—	—	—
28. xi. 36	46	3·85	3·85	12·89	12·86	—	—	—	—
29. xi. 36	46	4·05	4·10	13·01	12·99	—	—	—	—
30. xi. 36	48	4·15	4·05	12·93	12·95	—	—	—	—
1. xii. 36	46	3·65	4·00	12·32	12·88	41,600,000	+ + + + + +	4	– – – – – –
2. xii. 36	50	4·15	4·15	12·97	12·97	—	—	—	—
3. xii. 36	60	3·65	4·10	11·31	12·90	—	—	—	—
4. xii. 36	52	4·45	4·40	13·13	13·15	—	—	—	—
5. xii. 36	52	4·45	4·40	13·13	13·15	—	—	—	—
6. xii. 36	47	3·90	4·10	13·65	13·10	—	—	—	—
7. xii. 36	40	4·10	4·10	12·86	12·98	—	—	—	—
8. xii. 36	46	3·45	4·30	12·31	13·11	130,000	+ + + – – –	14	– – – – – –
9. xii. 36	47	4·15	4·20	12·74	12·95	80,000	+ + + – – –	Nil	– – – – – –
10. xii. 36	48	4·00	4·05	12·82	12·80	—	—	—	—
11. xii. 36	47	4·45	4·45	13·25	13·24	—	—	—	—
12. xii. 36	47	4·45	4·45	13·25	13·24	—	—	—	—
13. xii. 36	48	4·10	4·15	12·83	12·91	—	—	—	—
14. xii. 36	50	4·15	4·15	12·89	12·87	—	—	—	—
15. xii. 36	50	4·00	3·95	12·79	12·81	2,980,000	+ + + + – –	Nil	– – – – – –
16. xii. 36	50	4·20	4·25	12·98	13·02	—	—	—	—
17. xii. 36	48	4·15	4·25	12·88	12·92	2,420,000	+ + + + – –	60	– – – – – –
18. xii. 36	54	4·25	4·55	12·98	13·41	—	—	—	—
19. xii. 36	54	4·25	4·55	12·98	13·41	—	—	—	—
20. xii. 36	50	4·25	4·55	12·98	13·41	—	—	—	—
21. xii. 36	50	4·10	4·00	12·77	12·79	—	—	—	—
22. xii. 36	53	3·90	3·80	12·62	12·67	—	—	—	—
23. xii. 36	53	3·90	3·80	12·62	12·67	—	—	—	—
Average	49·6	3·96	3·99	12·80	12·84	—	—	—	—

* Plated on milk agar in 1/10,000, 1/100,000 and 1/1,000,000 dilutions.

† Coliform organisms present in 1 ml., 1/10 ml., 1/100 ml., 1/1000 ml., 1/10,000 ml., 1/100,000 ml.

‡ Plated on milk agar in 1/1, 1/10 and 1/100 dilutions.

§ Coliform organisms probably the result of improper sampling technique, sample not taken in Bacteriological Laboratory.

autoclave and heated to 212° F., when the bottles were closed with swing stoppers and heating continued to about 230° F. The bottles were held for a time at this temperature and were then air-cooled.

Before sterilization was begun samples of the cooled raw milk were taken and placed in sterilized Thermos flasks. These, packed in special boxes, were then sent to the Institute's Laboratory at Shinfield, Reading, together with crates of bottles of sterilized milk. The milks were despatched by passenger train immediately after completion of the sterilization process and were usually received in the Institute's Laboratory by about 1 p.m. Upon receipt in the Laboratory the raw milk was transferred to sterile Kilner jars and allowed to reach room temperature before it was used for feeding.

On Fridays a double supply of the milks was received and half of the raw milk was kept in Kilner jars in cold storage for use on Saturday. The milks received on Saturday were fed on Sunday, the raw milk being stored in a refrigerator. In order to safeguard against the non-arrival of milk on any day, sufficient of the previous day's milk was kept in the refrigerator overnight. With the exception of Saturdays and Sundays the milks were fed to the rats on arrival.

The temperature of the milk in each Thermos flask was noted as the flask was opened. It normally ranged from 45° to 55° F. Daily estimations of fat and total solids were carried out on the two types of milk and periodic bacteriological tests were also made. Table I gives the results of these tests. In 86 out of the 93 pairs of samples examined the differences in composition were very small, as shown in the Table. Probably owing to imperfect mixing seven pairs of samples showed differences varying between 0·20 and 0·85% in fat content and between 0·43 and 1·59% in total solids content.

Experiments with the raw and sterilized milks

The relative nutritive values of the two types of milk were compared in a series of studies which included: (1) the determination of the digestibility and biological values of the proteins, including a study of the effect of the method of feeding the milk on its biological value; (2) the vitamin A and carotene content; (3) the vitamin B complex and the vitamin B_1 and B_2 (flavin) potency; and (4) the vitamin C content of the raw and sterilized milks; (5) the total nutritive value of each type of milk was measured (*a*) by exclusive feeding of the milk supplemented with minerals, (*b*) and (*c*) by the feeding of limited amounts of milk in addition to a diet supplying energy, protein and salts, with and without yeast (as a source of the vitamin B complex), and (*d*) by exclusive feeding of milk supplemented with minerals and with energy in the form of sugar dissolved in the milk.

The first three studies are included in the present communication. Reports on the remaining investigations will appear in the next issue of this *Journal*.

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The milks used in these investigations were provided by United Dairies, Ltd., to whom our best thanks are due for their generosity and their helpful co-operation. We are especially indebted to their Chief Chemist, Mr E. B. Anderson, M.Sc., F.I.C., for helping us in every way and for organizing the supply of the milks.

We wish to express our gratitude to Prof. H. D. Kay for his constant help and support. Mr J. McClemont kindly carried out the bacteriological tests for us and we are indebted to Mr A. Wagstaff for the estimation of fat and total solids in the milks. Our best thanks are due to Miss D. V. Dearden for churning the butters.

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171. THE EFFECT OF COMMERCIAL STERILIZATION ON THE NUTRITIVE VALUE OF MILK

IIA. BIOLOGICAL VALUE AND DIGESTIBILITY OF THE PROTEINS (NITROGEN) OF MILK[1]

By K. M. HENRY and S. K. KON

Previous work from this laboratory (1) has shown that the biological value of the mixed proteins of milk is not lowered as a result of commercial pasteurization, that is to say of a comparatively mild heat treatment. The present experiments were carried out in order to determine the effect on milk proteins of the more drastic heat treatment involved in commercial sterilization.

Experimental

The method of Mitchell (2), as modified by Mitchell & Carman (3), was used for the determination of the biological value of the milk proteins.

(1) *Technique*

Six pairs of littermate female rats from 4 litters were placed, when 24–25 days old and weighing approximately 60–68 g., in metabolic cages made of glass and stainless steel. A full description of the cages, of the method of feeding and of the methods of collecting and of analysing the excreta and milks, will be found in our previous paper (1).

(2) *Diets*

The composition of the low egg-nitrogen and "nitrogen-free" diets was the same as previously used (1). Their nitrogen and moisture contents are given in Table I.

Table I. *Nitrogen and moisture contents of the experimental diets*

Diet	% N	% moisture
Low egg-nitrogen	0·646	9·79
"Nitrogen-free"	0·0375	9·59

The milks were fed at a 7·8% level of protein (N × 6·38) intake. In the previous work, to which reference has already been made, the milk was fed separately from the basal "nitrogen-free" diet which was mixed with half of its weight of water. At the start of the first milk period in the present experiment the same method was used but difficulty was experienced in persuading

[1] Read in part before the Biochemical Society, 15 January 1937. (Henry & Kon, 1937, *J. Soc. Chem. Ind., Lond.*, **56**, 97.)

the rats to drink all of their milk. This was particularly noticeable with the rats receiving the sterilized milk. We found that by using some of the milk instead of water to make the basal diet into a paste the balance of the milk could then be fed separately, and was generally consumed quantitatively. By this method about 20% of the milk was fed mixed with the diet. The intakes of milk and diet were equalized within pairs as far as possible. Throughout the experiment each rat received daily 0·3 ml. of an Osborne-Wakeman (4) aqueous extract of brewer's yeast equivalent to 0·3 g. of dried yeast and containing 4·2 mg. of nitrogen. The nitrogen from this source was disregarded in the subsequent calculations. The rats also received daily 2 drops of cod-liver oil.

Table II gives the daily analyses of the experimental milks.

Table II. *Analyses of milks* (*g. of nitrogen*/100 *ml. of milk*)

1st experimental period			2nd experimental period		
Date	Raw	Sterilized	Date	Raw	Sterilized
10. x. 36	0·563	0·558	20. x. 36	0·554	0·560
11. x. 36	0·567	0·555	21. x. 36	0·579	0·581
12. x. 36	0·567	0·569	22. x. 36	0·571	0·565
13. x. 36	0·563	0·565	23. x. 36	0·555	0·554
14. x. 36	0·558	0·558	24. x. 36	0·555	0·554
15. x. 36	0·569	0·568	25. x. 36	0·563	0·561

(3) *Results*

The results of the experiment are given in Tables III, IV and V. Table III gives individual data. Table IV gives the average figures, together with their standard deviations and standard errors of the mean, for the biological values, the true digestibilities, and the "protein values" of the milks, for each experimental period. It also gives the combined values for the two experimental periods for the raw and sterilized milks. Table V shows the tests of statistical significance. A description of the statistical methods employed will be found elsewhere (5). The *t*-table of "Student" (6) was used.

During the first milk period rat No. 8 on sterilized milk consistently left most of the milk which had not been mixed with the diet, the figures obtained for this rat and for its littermate are therefore omitted from the average values and from the statistical tests. During the second period another rat on sterilized milk, No. 5, left considerable amounts of milk, this rat and its littermate are also omitted from the averages.

The results given in Table IV show a slight difference in the true digestibility of the proteins between raw and sterilized milk. The apparent lowering from 96·4 to 95·3 is, however, without statistical significance (Table V). On the other hand the biological value of the proteins has been lowered by about 6%. Reference to Table V shows that this difference is not quite significant for the first experimental period, the odds against the difference being due to errors of sampling being 17 : 1. In the second period and for the combined values the odds rise to 312 : 1 and 625 : 1 respectively, showing that

Table III. *Nitrogen metabolism; individual data for 6-day periods*

Rat no.	Type of milk	Initial wt. g.	Final wt. g.	Food intake: Diet g.	Food intake: Milk ml.	Food intake: Total* solids g.	N intake mg.	Faecal N mg.	Body N in faeces mg.	Food N in faeces mg.	Absorbed N mg.	N in urine mg.	Body N in urine mg.	Food N in urine mg.	Food N retained mg.	Biological value	True digestibility	"Protein value" of fresh milk
								Low egg-nitrogen diet										
1	—	60	63	43·95	—	—	—	91·00	2·07†	—	—	74·30	119·84‡	—	—	—	—	—
2	—	60	62	41·91	—	—	—	86·15	2·08†	—	—	78·80	129·18‡	—	—	—	—	—
3	—	63	71	55·22	—	—	—	110·50	2·00†	—	—	90·95	135·75‡	—	—	—	—	—
4	—	65	71	55·13	—	—	—	106·60	1·93†	—	—	88·95	130·81‡	—	—	—	—	—
5	—	68	71	51·60	—	—	—	111·25	2·16†	—	—	87·35	124·79‡	—	—	—	—	—
6	—	67	73	52·50	—	—	—	102·30	1·95†	—	—	92·10	131·57‡	—	—	—	—	—
7	—	64	65	40·43	—	—	—	97·35	2·41†	—	—	84·10	129·38‡	—	—	—	—	—
8	—	65	65	36·51	—	—	—	94·50	2·59†	—	—	86·65	133·31‡	—	—	—	—	—
9	—	69	73	48·34	—	—	—	97·10	2·01†	—	—	81·10	114·23‡	—	—	—	—	—
10	—	65	69	48·50	—	—	—	110·15	2·27†	—	—	85·60	127·76‡	—	—	—	—	—
11	—	64	65	43·08	—	—	—	105·65	2·45†	—	—	78·45	120·69‡	—	—	—	—	—
12	—	65	68	45·99	—	—	—	96·15	2·09†	—	—	83·40	124·48‡	—	—	—	—	—
								1st milk period										
1	Raw	64	74	32·26	84·69	44·51	490·10	107·60	101·04	6·56	483·54	160·45	79·16	81·29	402·25	83·2	98·7	2·77
2	Sterilized	60	75	32·41	86·89	45·40	500·93	109·45	98·52	10·93	490·00	178·75	81·36	97·39	392·61	80·1	97·8	2·63
3	Raw	70	80	33·39	89·00	46·25	514·94	107·05	99·44	7·61	507·33	156·40	94·04	62·36	444·97	87·7	98·5	2·94
4	Sterilized	68	71	27·01	64·02	36·26	370·40	100·90	74·33	26·57	343·83	164·65	82·17	82·48	261·35	76·0	92·8	2·36
5	Raw	68	79	30·39	81·00	42·10	468·67	99·05	95·57	3·48	465·19	153·35	83·83	69·52	395·67	85·1	99·3	2·86
6	Sterilized	68	76	28·14	75·00	38·98	432·48	82·75	77·96	4·79	427·69	187·35	87·60	99·75	327·94	76·6	98·9	2·56
§ 7	Raw	70	82	39·00	103·99	54·03	601·63	143·60	108·31	35·29	566·34	168·90	90·56	78·34	488·00	86·2	94·1	2·72
8	Sterilized	54	54	22·50	21·76	25·65	130·77	88·70	70·28	18·42	112·35	140·85	69·48	71·37	40·98	36·5	85·9	0·90
9	Raw	77	90	39·00	103·91	54·02	601·18	158·90	119·92	38·98	562·20	153·45	92·34	61·11	501·09	89·1	93·5	2·82
10	Sterilized	66	73	30·39	80·90	42·09	466·50	135·50	100·60	34·90	431·60	162·35	84·83	77·52	354·08	82·0	92·5	2·54
11	Raw	70	80	36·12	94·01	49·71	544·08	150·10	119·30	30·80	513·28	183·15	90·05	93·10	420·18	81·9	94·3	2·59
12	Sterilized	71	80	34·14	91·00	47·29	524·55	135·05	105·93	29·12	495·43	172·65	92·46	80·19	415·24	83·8	94·4	2·66

2nd milk period																		
1	Sterilized	81	87	34·87	92·99	48·34	536·19	140·55	117·47	23·08	513·11	193·60	93·06	100·54	412·57	80·4	95·7	2·56
2	Raw	82	89	36·77	98·00	51·00	566·03	137·60	114·24	23·36	542·67	192·60	96·53	96·07	446·60	82·3	95·9	2·66
3	Sterilized	79	86	30·39	81·00	42·12	466·62	112·10	96·45	15·65	450·97	174·05	97·47	76·58	374·39	83·0	96·6	2·69
4	Raw	75	90	37·87	100·99	52·54	582·58	129·65	111·91	17·74	564·84	152·40	88·87	63·53	501·31	88·8	97·0	2·92
§ 5	Sterilized	82	81	24·37	57·25	32·66	331·29	93·95	76·75	17·20	314·09	189·75	85·74	104·01	210·08	66·9	94·8	2·09
6	Raw	85	99	44·25	118·00	61·39	680·71	127·00	125·24	1·76	678·95	198·00	104·92	93·08	585·87	86·3	99·7	2·92
7	Sterilized	90	92	36·97	98·92	51·31	570·84	136·90	128·28	8·62	562·22	235·55	101·28	134·27	427·95	76·1	98·5	2·49
8	Raw	55	63	28·14	75·00	39·03	432·49	113·05	111·24	1·81	430·68	160·85	73·78	87·07	343·61	79·8	99·6	2·63
9	Sterilized	94	102	43·48	115·98	60·29	668·58	176·30	144·09	32·21	636·37	244·95	104·50	140·45	495·92	77·9	95·2	2·47
10	Raw	83	86	34·52	92·00	47·88	531·19	159·00	119·22	39·78	491·41	168·95	94·87	74·08	417·33	84·9	92·5	2·62
11	Sterilized	79	84	33·35	88·98	46·25	513·19	167·20	117·01	50·19	463·00	210·85	98·06	112·79	350·21	75·6	90·2	2·25
12	Raw	84	91	41·63	110·99	57·74	640·35	169·85	135·69	34·16	606·19	224·10	105·14	118·96	487·23	80·4	94·7	2·55
Low egg-nitrogen diet																		
1	—	97	103	61·25	—	—	—	157·95	2·58†	—	—	106·85	106·85‡	—	—	—	—	—
2	—	98	103	58·75	—	—	—	136·00	2·31†	—	—	105·95	104·90‡	—	—	—	—	—
3	—	98	104	62·25	—	—	—	149·15	2·40†	—	—	110·55	109·46‡	—	—	—	—	—
4	—	101	110	67·59	—	—	—	151·45	2·24†	—	—	102·55	96·75‡	—	—	—	—	—
5	—	93	102	59·91	—	—	—	145·65	2·43†	—	—	93·85	95·77‡	—	—	—	—	—
6	—	108	117	70·35	—	—	—	146·35	2·08†	—	—	120·25	106·42‡	—	—	—	—	—
7	—	102	116	80·65	—	—	—	204·65	2·54†	—	—	112·75	103·44‡	—	—	—	—	—
8	—	69	72	40·20	—	—	—	119·20	2·97†	—	—	86·25	121·48‡	—	—	—	—	—
9	—	112	119	75·83	—	—	—	193·00	2·55†	—	—	119·85	103·32‡	—	—	—	—	—
10	—	98	102	69·06	—	—	—	177·95	2·58†	—	—	111·05	111·05‡	—	—	—	—	—
11	—	94	98	55·27	—	—	—	141·95	2·57†	—	—	114·35	119·11‡	—	—	—	—	—
12	—	99	109	73·13	—	—	—	180·90	2·47†	—	—	122·00	117·31‡	—	—	—	—	—

* For the calculation of the total solid intake, the solids of milk were measured and the figure was then corrected to the same moisture content as that of the basal diet.

† These figures represent the metabolic faecal nitrogen per g. of food consumed and are used in estimating the body N in the faeces in the intermediate periods from the amounts of food consumed.

‡ These figures represent the endogenous urinary nitrogen per 100 g. of body weight and are used in estimating the body N in the urine in the intermediate periods from the average body weight.

§ Omitted from averages.

Table IV. *Average figures for the biological values, true digestibilities and "protein values" of the mixed proteins* (N × 6·38) *of raw and sterilized milks*

Raw milk			Sterilized milk		
Biological value	True digestibility	"Protein value"	Biological value	True digestibility	"Protein value"
		1st experimental period			
85·40	96·86	2·796	79·70	95·28	2·550
S.D. ± 3·01	± 2·73	±0·131	± 3·38	± 2·92	±0·117
S.E.M. ± 1·35	± 1·22	±0·059	± 1·51	± 1·31	±0·052
		2nd experimental period			
83·24	95·94	2·676	78·60	95·24	2·492
S.D. ± 3·69	± 2·64	±0·142	± 3·10	± 3·09	±0·151
S.E.M. ± 1·65	± 1·18	±0·064	± 1·38	± 1·38	±0·072
		Pooled values for 1st and 2nd experimental periods			
84·32	96·40	2·736	79·15	95·26	2·521
S.D. ± 3·37	± 2·58	±0·144	± 3·18	± 2·82	±0·136
S.E.M. ± 1·07	± 0·82	±0·045	± 0·98	± 0·90	±0·043

Table V. *Statistical significance of the differences in biological values, true digestibilities and "protein values" between raw and sterilized milks*

	Biological value	True digestibility	"Protein value"
	1st experimental period		
Average for raw milk	85·40	96·86	2·796
Average for sterilized milk	79·70	95·28	2·550
Difference in percentage units (raw-sterilized)	+ 5·70	+ 1·58	+0·246
S.E.M.	± 2·349	± 1·048	±0·1065
*P**	1 : 17	1 : 4	1 : 15
	Not significant	Not significant	Not significant
	2nd experimental period		
Average for raw milk	83·24	95·94	2·676
Average for sterilized milk	78·60	95·24	2·492
Difference in percentage units (raw-sterilized)	+ 4·64	+ 0·70	+0·184
S.E.M.	± 0·869	± 1·151	±0·0359
*P**	1 : 312	1 : 2	1 : 263
	Significant	Not significant	Significant
	Pooled values for 1st and 2nd experimental periods		
Average for raw milk	84·32	96·40	2·736
Average for sterilized milk	79·15	95·26	2·521
Difference in percentage units (raw-sterilized)	+ 5·17	+ 1·14	+0·215
S.E.M.	± 1·195	± 0·749	±0·0539
*P**	1 : 625	1 : 6	1 : 357
	Significant	Not significant	Significant

* *P* = Probability that a difference at least as great as the observed difference would have arisen by random sampling from a homogeneous population.

the differences are definitely significant. The "protein value" of the milk calculated according to Mitchell(7) is also lowered by about 8% as a result of sterilization. Here again the differences are hardly significant for the first milk period, but are definitely so for the second period and for the pooled values.

It can, therefore, be said with confidence that while the digestibility of the proteins of milk is unaffected by commercial sterilization the heat treatment has injured one or more of the essential amino acids. Our experiments give no clue to the identity of the affected amino acid. If the work of Fairbanks & Mitchell(8) on skim milk powders can be taken as a guide, it would seem that the decline in biological value is due to a partial destruction of cystine. As Jackson & Block(9) have shown that, under certain conditions, methionine and cystine can replace each other, it is also possible that only the former amino acid was affected or that the two had jointly suffered some loss.

We pointed out in our previous paper(1) that the biological value of 80·6% for raw liquid milk was much lower than the figure of 90·0% obtained by Fairbanks & Mitchell(8) for liquid skim milk. It was suggested that this difference might be ascribed to the method of feeding. In our experiments the milk was fed separately from the basal diet, while Fairbanks & Mitchell probably incorporated the milk in the diet. The present experiment in which 20% of the milk was mixed with the diet appears to bear out our suggestion, as the biological value of the proteins of the raw liquid milk has risen to 84·3.

We have investigated the problem further in a separate experiment which is described in the Note following(10).

SUMMARY

1. The biological values and true digestibilities of the proteins of raw and commercially sterilized milks from the same bulk have been compared on rats by the method of Mitchell.

2. For raw milk a biological value of 84·3 and a true digestibility of 96·4 were obtained, while the figures for sterilized milk were 79·1 and 95·3 respectively.

3. Statistical tests showed that the difference in true digestibility was not significant but that the lowering of the biological value by about 6% could be ascribed to the effect of the heat treatment, the odds against chance factors being 625 : 1.

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172. THE EFFECT OF COMMERCIAL STERILIZATION ON THE NUTRITIVE VALUE OF MILK

IIB. A NOTE ON THE EFFECT OF THE METHOD OF FEEDING DRIED SKIMMED MILK ON THE BIOLOGICAL VALUE OF ITS PROTEINS

By K. M. HENRY, E. W. IKIN and S. K. KON

We suggested in a previous paper(1) that when the biological value of the proteins of liquid milk is measured by a metabolic method the way in which the milk is fed may have an effect on the result.

Subsequent work carried out by us(2) seemed to support this suggestion.

To put the matter to a direct test the work described below was carried out. It differs from our previous experiments in that spray dried skimmed milk instead of whole liquid milk was used. This was done because we found it very difficult to mix the necessary amounts of liquid milk and basal diet without the constituents separating on standing. Should a rat fail to consume the mixture completely the components would not be taken in a fixed ratio, which would greatly complicate the measurement of nitrogen intake.

Experimental

Six pairs of 26 days old littermate female rats from three litters, weighing 48–68 g. were placed in our usual nitrogen-metabolism cages(1). They were allotted by toss of coin to receive either dried skimmed milk fed mixed with the basal diet or dried skimmed milk fed separately from the basal diet. Details of the basal diets and experimental technique have been described in a previous paper(1). The mixed diet consisted of 16·67 parts of skimmed dried milk and 83·33 parts of the basal "nitrogen free" diet.

When fed separately the two constituents were offered in the same ratio. As far as possible the intakes of food were equalized within pairs. Values for both methods of feeding were obtained on each rat. The milk was fed at a 6·9% level of protein (N × 6·38) intake.

Table I gives the initial analyses, for nitrogen and moisture, of the experimental diets.

Table I. *Analyses of the experimental diets*

Diet	% N	% moisture
Low egg-nitrogen	0·650	9·73
"Nitrogen-free"	0·040	9·79
"Nitrogen-free" + dried milk	0·973	8·96
Dried milk	5·750	5·15

Moisture determinations were made on the experimental diets at frequent intervals. All results were worked out on a moisture-free basis. For feeding, the diets and milk were mixed to a paste with distilled water. Vitamins were as usual supplied by 0·3 ml. of an Osborne-Wakeman (3) aqueous yeast extract and 2 drops of cod-liver oil daily.

Results

Table II gives the individual data for the experiment. Average figures for the biological values and for the true digestibilities of the milk for both methods of feeding are shown in Table III. Values for both experimental periods and also the combined values for the two periods are given. A statistical treatment of the average differences is also given in Table III. During the first half of the first milk period rat No. 8, which was given milk separately from the basal diet, consistently left its milk. The figures for this animal and for its littermate are therefore omitted from the averages.

It will be seen from Table III that the true digestibility of dried separated milk is very slightly higher when the milk is fed separately from the basal diet than when incorporated with the diet. The difference, however, is not statistically significant.

Turning now to the effect of the method of feeding on the biological value of the milk, it will be seen from Table III that although higher values are obtained when the milk is fed incorporated with the diet, the differences are without statistical significance whether the two experimental periods are considered separately or together. The difference is quite insignificant for the first experimental period, and individual values, especially for the rats on separate feeding, fluctuate widely. The variations were much smaller in the second period, where the difference more nearly approaches statistical significance. When the values for the two experimental periods are pooled, the difference in favour of the mixed diet with odds of 9:1 is still without statistical significance. In the first experimental period an abnormally high value of 98·2 was obtained for rat No. 2 which was given the milk separately from the diet. If the figures for this rat and its littermate are omitted both from the first experimental period and from the pooled values we get the following results: for the 1st period the difference in percentage units is $+3{\cdot}3$, S.E.M. $=2{\cdot}30$, $P=1:5$ in favour of mixed feeding: for the pooled values the difference in percentage units is $+4{\cdot}13$, S.E.M. $=\pm 1{\cdot}59$, $P=1:37$. Thus while omission of this rat only slightly increases the statistical significance for the first period, when the pooled values are considered definite statistical significance is reached. On experimental grounds there is no reason for omitting this animal.

The average biological value of 90 % obtained when the dried whole milk is incorporated in the diet agrees well with the figure obtained by Fairbanks & Mitchell (4) for skim milk fed raw or after drying at low temperature. The figure of 86 or 87 % obtained by us when the milk is fed separately from the

Table II. *Nitrogen metabolism; individual data for 6-day periods*

Rat no.	Method of feeding milk	Initial wt. g.	Final wt. g.	Food intake* (g.) Diet	Milk	Total solids	N intake mg.	Faecal N mg.	Body N in faeces mg.	Food N in faeces mg.	Absorbed N mg.	N in urine mg.	Body N in urine mg.	Food N in urine mg.	Food N retained mg.	Biological value	True digestibility
								Low egg-nitrogen diet									
1	—	68	70	43·77	—	—	—	72·10	1·65†	—	—	110·55	160·3‡	—	—	—	—
2	—	62	67	42·75	—	—	—	92·15	2·16†	—	—	97·10	149·4‡	—	—	—	—
3	—	62	65	47·80	—	—	—	115·40	2·41†	—	—	101·10	158·0‡	—	—	—	—
4	—	60	67	49·13	—	—	—	110·45	2·25†	—	—	99·25	155·1‡	—	—	—	—
5	—	50	54	38·11	—	—	—	92·50	2·43†	—	—	90·15	173·4‡	—	—	—	—
6	—	49	53	36·42	—	—	—	97·10	2·67†	—	—	87·00	170·6‡	—	—	—	—
§ 7	—	59	64	44·50	—	—	—	99·30	2·23†	—	—	93·80	151·3‡	—	—	—	—
8	—	59	64	46·02	—	—	—	117·00	2·54†	—	—	89·20	143·9‡	—	—	—	—
9	—	63	67	46·33	—	—	—	112·05	2·42†	—	—	103·30	159·0‡	—	—	—	—
10	—	59	63	44·61	—	—	—	109·85	2·46†	—	—	91·60	150·2‡	—	—	—	—
11	—	60	64	43·47	—	—	—	122·95	2·83†	—	—	93·90	151·5‡	—	—	—	—
12	—	61	64	44·67	—	—	—	115·20	2·58‡	—	—	92·05	146·1‡				
								1st milk period									
1	In diet	72	83	44·52	—	44·52	475·90	116·70	82·38	34·32	441·6	154·00	113·6	40·4	401·2	90·8	92·8
2	Separately	69	79	35·65	7·56	43·21	472·09	116·70	100·70	16·00	456·1	108·30	100·0	8·3	447·8	98·2	96·6
3	In diet	67	76	42·86	—	42·86	458·10	112·15	101·10	11·05	447·1	151·70	109·5	42·2	404·9	90·6	97·6
4	Separately	66	76	36·01	7·49	43·50	468·24	98·85	100·90	0·00	468·2	164·35	100·7	63·7	404·5	86·3	100·0
5	In diet	56	68	45·21	—	45·21	483·20	125·00	113·40	11·60	471·6	122·70	96·9	25·8	445·8	94·6	97·6
6	Separately	55	59	24·69	5·33	30·02	332·66	90·65	78·05	12·60	320·1	102·45	89·3	13·2	306·9	95·9	96·2
§ 7	In diet	65	77	47·20	—	47·20	504·50	162·75	106·70	56·05	448·5	142·70	98·4	44·3	404·2	90·1	88·9
8	Separately	58	63	24·77	5·09	29·86	318·20	100·85	74·06	26·79	291·4	165·55	80·4	85·2	206·2	70·8	91·6
9	In diet	67	75	41·80	—	41·80	446·80	138·45	106·60	31·85	415·0	147·90	107·0	40·9	374·1	90·2	92·9
10	Separately	65	72	33·50	7·17	40·67	447·64	142·05	100·00	42·05	405·6	137·70	93·9	43·8	361·8	89·2	90·6
11	In diet	64	75	47·23	—	47·23	504·80	132·45	132·20	0·25	504·6	144·45	103·0	41·5	463·1	91·8	100·0
12	Separately	64	73	39·05	8·11	47·16	506·88	129·30	119·30	10·00	496·9	185·05	98·1	87·0	409·9	82·5	98·0

2nd milk period																	
1	Separately	84	96	42·41	8·82	51·23	551·26	143·15	104·50	38·65	512·6	197·55	117·8	79·8	432·8	84·4	93·0
2	In diet	80	92	50·90	—	50·90	544·10	142·55	127·70	14·85	529·3	171·70	104·0	67·7	461·6	87·2	97·3
3	Separately	76	77	28·51	5·89	34·40	368·14	99·45	79·47	19·98	348·2	193·20	112·6	80·6	267·6	76·6	94·6
4	In diet	80	88	42·69	—	42·69	456·20	126·60	102·10	24·50	431·7	150·25	107·8	42·5	389·2	90·1	94·6
5	Separately	70	75	33·41	6·94	40·35	433·80	121·25	104·90	16·35	417·5	150·25	101·4	48·9	368·6	88·3	96·2
6	In diet	65	74	40·42	—	40·42	432·00	122·15	102·30	19·85	412·2	123·75	99·8	24·0	388·2	94·2	95·4
7	Separately	70	82	32·67	6·80	39·47	424·97	114·35	90·00	24·35	400·6	159·50	95·5	64·0	336·6	84·0	94·3
8	In diet	66	78	42·69	—	42·69	456·20	141·80	103·30	38·50	417·7	125·10	86·4	38·7	379·0	90·7	91·5
9	Separately	74	86	42·79	8·89	51·68	555·62	173·40	138·00	35·40	520·2	189·95	114·0	76·0	444·2	85·4	93·6
10	In diet	74	86	51·41	—	51·41	549·50	169·30	127·00	42·30	507·2	156·80	97·5	59·3	447·9	88·3	92·3
11	Separately	78	90	43·26	8·97	52·23	560·74	185·40	144·20	41·20	519·5	177·80	120·1	57·7	461·8	88·9	92·7
12	In diet	76	87	52·69	—	52·69	563·10	170·95	130·20	40·75	522·4	190·55	113·4	77·2	445·2	85·2	92·8
Low egg-nitrogen diet																	
1	—	104	114	63·19	—	—	—	141·70	2·24†	—	—	126·70	116·2‡	—	—	—	—
2	—	98	99	42·86	—	—	—	114·90	2·68†	—	—	105·60	106·7‡	—	—	—	—
3	—	87	88	35·62	—	—	—	80·45	2·26†	—	—	123·40	140·3‡	—	—	—	—
4	—	96	106	63·09	—	—	—	154·95	2·46†	—	—	116·20	115·0‡	—	—	—	—
5	—	85	90	46·00	—	—	—	123·45	2·68†	—	—	107·15	121·7‡	—	—	—	—
6	—	78	80	38·81	—	—	—	95·30	2·46†	—	—	101·60	128·6‡	—	—	—	—
7	—	91	101	57·31	—	—	—	132·65	2·31†	—	—	108·40	112·9‡	—	—	—	—
8	—	88	98	58·50	—	—	—	138·10	2·36†	—	—	100·45	108·0‡	—	—	—	—
9	—	96	102	59·57	—	—	—	166·70	2·80†	—	—	132·80	134·2‡	—	—	—	—
10	—	95	99	52·97	—	—	—	130·85	2·47†	—	—	104·55	107·8‡	—	—	—	—
11	—	91	98	54·40	—	—	—	148·70	2·73†	—	—	131·70	138·7‡	—	—	—	—
12	—	93	100	59·24	—	—	—	143·30	2·42†	—	—	130·40	134·4‡	—	—	—	—

* On the dry basis.

† These figures represent the metabolic faecal nitrogen per g. of food consumed and are used in estimating the body N in the faeces in the intermediate periods from the amounts of food consumed.

‡ These figures represent the endogenous urinary nitrogen per 100 g. of body weight and are used in estimating the body N in the urine in the intermediate periods from the average body weight.

§ Omitted from averages.

Table III. *Average values for and statistical significance of the differences in the biological values and the true digestibilities of the mixed proteins* (N × 6·38) *of dried skimmed milk fed in or separately from a "nitrogen-free" diet*

	1st experimental period	2nd experimental period	Pooled values for 1st and 2nd periods
	Biological value		
Average *A*. Milk fed in diet	91·60 ± 0·79	89·28 ± 1·28	90·34 ± 0·76
Average *B*. Milk fed separately from diet	90·44 ± 2·91	84·60 ± 1·80	87·25 ± 1·81
Difference in percentage units (*A*-*B*)	+1·16	+4·68	+3·09
S.E.M.	±2·78	±2·31	±1·78
*P**	1 : 1	1 : 11	1 : 9
	Not significant	Not significant	Not significant
	True digestibility		
Average *A*. Milk fed in diet	96·18 ± 1·43	93·98 ± 0·89	94·98 ± 0·84
Average *B*. Milk fed separately from diet	96·28 ± 1·57	94·07 ± 0·53	95·07 ± 0·80
Difference in percentage units (*A*-*B*)	−0·10	−0·09	−0·09
S.E.M.	±1·25	±0·98	±0·74
*P**	1 : 1	1 : 1	1 : 1
	Not significant	Not significant	Not significant

* *P* = Probability that a difference at least as great as the observed difference would have arisen by random sampling from a homogeneous population.

diet is considerably higher than the value of 81% obtained previously in this laboratory (3) for raw whole milk fed separately from the basal diet.

Thus, although the present experiment suggests, but does not prove, that the biological value of the mixed proteins of dried milk may be altered by the method of feeding the milk, it fails to explain the low figure obtained by us when raw milk was fed separately from the basal diet. It should be remembered that in one case the milk was dried, in the other liquid.

Summary

1. In experiments in which the biological value of the proteins was measured spray dried skimmed milk was fed separately from or mixed with a basal "nitrogen free" diet.

2. At a 6·9% level of protein (N × 6·38) intake the first method of feeding yielded a biological value of 87·2, the second of 90·3. The difference was not statistically significant (odds of 9 : 1 against chance factors).

3. The findings do not account for the low biological value reported by us previously for the proteins of raw liquid milk.

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173. THE EFFECT OF COMMERCIAL STERILIZATION ON THE NUTRITIVE VALUE OF MILK

III. EFFECT ON THE VITAMIN A AND CAROTENE CONTENT OF MILK

By A. E. GILLAM, K. M. HENRY, S. K. KON and P. WHITE

During the course of the experiments on sterilized milk typical samples of milk fat were obtained from both raw and sterilized milk and examined once or twice each week. As in previous experiments(1), colorimetric tests were carried out at Shinfield (K. M. H. & S. K. K.) and spectrophotometric tests at Manchester (A. E. G.). One of us (P. W.) was responsible for the statistical treatment of the results.

Experimental

As the sterilized milk had been homogenized it was impossible to churn it. The following method for the extraction of the fat, was therefore used: One litre of milk (500 ml. from each of two consecutive days' supply) was put in a large separating funnel, 700 ml. ether were added, followed successively by 50 ml. alcohol, 25 ml. KOH (sp. gr. 1·26–1·27) and a further 150 ml. alcohol. After each addition the mixture was well shaken. Rapid separation occurred, after which the aqueous layer was drawn off and further extracted with 400, 200 and 200 ml. lots of ether respectively. The combined ether extracts were then washed three times with 250 ml. of water and dried over anhydrous sodium sulphate. The ether was removed and the fat weighed before storing at $-2°$ C. until analysed. Corresponding samples of raw milk were extracted and treated in the same way. In addition, butter fat was churned from the raw milk in the customary fashion to compare the stability of carotene and vitamin A in the two processes. The results of this comparison will be discussed in a separate communication. The methods of analysis have been fully described in a previous publication(1), the only departure from the procedure there recorded being a duplication of all the spectrophotometric determinations.

Results

The data for carotene are shown in Table I.

From the colorimetric measurements it appears that sterilization brings about a loss of only 2·4% (0·49 units) in the carotene content but that this loss is just significant ($P = 1:23$). The spectrophotometric method shows no significant effect of sterilization, the difference observed (an increase of

Table I. *Carotene*

Colorimetric method, Moore yellow units per g. of fat			Spectrophotometric method, mg. per 100 g. of fat*		
Raw	Sterilized	Difference raw-sterilized	Raw	Sterilized	Difference raw-sterilized
20·2	19·7	+0·5	$0{\cdot}83_5$	$0{\cdot}89_5$	−0·06
20·9	20·6	+0·3	0·89	0·81	+0·08
22·0	20·6	+1·4	0·83	0·93	−0·10
21·8	19·7	+2·1	$0{\cdot}86_3$	$0{\cdot}81_0$	+0·05
21·5	21·3	+0·2	0·86	$0{\cdot}81_5$	+0·04
21·5	21·5	0	0·81	$0{\cdot}77_1$	+0·04
20·4	20·4	0	$0{\cdot}78_5$	$0{\cdot}86_5$	−0·08
21·5	20·6	+0·9	0·77	$0{\cdot}78_7$	−0·02
19·5	19·5	0	$0{\cdot}74_5$	$0{\cdot}76_8$	−0·02
20·2	20·2	0	0·78	—	—
12·9	12·9	0	$0{\cdot}58_1$	0·56	+0·02
Mean difference		+0·49 (+2·4%)			$-0{\cdot}004_5$ (−0·6%)
Standard error of the mean		±0·21 (1·0%)			±0·019 (2·4%)
		P†=1 : 23 Significant			P†=1 : 1 Not significant

* These figures are calculated on the basis of $E^{1\%}_{1\,\text{cm.}} = 2200$ for pure carotene in $CHCl_3$ at 463 mμ(3).

† P = probability that a difference at least as great as the observed difference would have arisen by random sampling from a homogeneous population.

$0{\cdot}004_5$ mg. or 0·6%) being much smaller than its own standard error. The latter result is not inconsistent with the former since the mutual agreement of the spectrophotometric differences is somewhat less than that of the colorimetric differences. As the standard error of the mean spectrophotometric difference is 2·4% of the average reading in this experiment, a mean difference of less than about 5·5% could not have been returned as significant by this method without the use of more than eleven pairs of samples. It is therefore not to be expected that the mean difference of 2·4% just revealed by the colorimetric method should have been confirmed by the spectrophotometric method.

The results of the vitamin A determinations are contained in Table II.

The colorimetric method shows a small loss (0·8%) of vitamin A on sterilization, but as this loss is no greater than its own standard error, it is not significant. It would have been necessary for the effect to have been as great as 2% for it to have been detected with confidence by an experiment of this type, involving eleven pairs of samples and having the same range of random error as that occurring in the present experiment.

The spectrophotometric method at first sight shows a considerable gain (10%) in vitamin A on sterilization, but the standard error of this figure is as high as 7% and the apparent gain, though suggestive, cannot be taken as significant (P=1 : 5). The method appears on statistical grounds to involve some inherent source of error, particularly when applied to vitamin A determinations on heated milks. Occasional readings (omitted from table) are obtained which are disproportionately great in relation to the colorimetric

Table II. *Vitamin A*

Colorimetric method, Moore blue units per g. of fat			Spectrophotometric method, mg. per 100 g. of fat*		
Raw	Sterilized	Difference raw-sterilized	Raw	Sterilized	Difference raw-sterilized
57	59	−2	1·71	—	—
60	60	0	—	1·50	—
64	60	+4	1·52	1·79	−0·27
62	60	+2	1·34	1·35	−0·01
62	62	0	1·33	1·37	−0·04
62	62	0	1·46	1·87	−0·41
60	60	0	1·29	1·73	−0·44
62	62	0	1·37	1·48	−0·11
62	62	0	1·25	1·42	−0·17
58	58	0	1·30	—	—
51·3	50	+1·3	1·35	1·04	+0·31
Mean difference		+0·5 (+0·8%)			−0·14 (−10%)
Standard error of the mean		±0·5 (0·8%)			±0·10 (7%)
		P†=1 : 3 Not significant			P†=1 : 5 Not significant

* Based on the assumption that vitamin A itself has an intensity of absorption at 328 mμ $E^{1\%}_{1\,cm.}$ = 1600 (in alcohol).

† P = probability that a difference at least as great as the observed difference would have arisen by random sampling from a homogeneous population.

values of the same specimens. There are no corresponding very small readings. This would suggest that the high standard deviation of the results is not due to the co-operation of many independent sources of small errors, but that there is a source of error which in many cases does not operate at all, but which, if it does operate, can only do so in such a way as to give rise to a large increase in the reading.

In view of these results it should be clearly understood that absorption of light near 328 mμ is not a specific test for vitamin A, although under certain conditions it is very nearly so. The discrepancies just mentioned are almost certainly due to substances in the unsaponifiable matter of the milk fat which, during saponification and subsequent extraction, are liable to undergo change with the production of compounds exhibiting general absorption in the region 300–350 mμ. The occasional development of non-vitamin A absorption at 328 mμ in the unsaponifiable matter of milk fat has already been reported by other workers (2) and our experience further shows that the heating of milk renders the unsaponifiable matter even more susceptible to these possibly oxidative changes. However, careful consideration of the combined colorimetric and spectrophotometric results shows that the sterilization of milk under the conditions specified causes no significant loss of vitamin A.

Effect of storage on the vitamin A and carotene content of sterilized milk

As sterilized milk can be, and sometimes is, stored for several weeks, we investigated the effects of such storage on the vitamin A and carotene content of the milk. For this purpose duplicates of some of the samples were kept in a

cool dark place for a month or six weeks and the fat was then extracted and analysed as usual. The results are given in Table III. In no case was any significant effect of storage detected. The mutual consistency of the results was such that an effect might have been detected by the colorimetric method if it had been as great as 5% for carotene and 3½% for vitamin A, but each of the differences observed was too small to be significant according to the usual statistical tests.

In the case of the spectrophometric measurements on vitamin A the results suggest a loss on storage, but the level of significance ($P = 1 : 10$) is too low for the suggestion to be received with any confidence.

Table III. *The effect of storage on the vitamin A and carotene of sterilized milk*

Carotene

Colorimetric method, Moore yellow units per g. fat			Spectrophometric method, mg. per 100 g. fat*		
Kept	Original	Difference kept-original	Kept	Original	Difference kept-original
20·8	20·6	+0·2	0·74	0·79	−0·05
19·1	20·2	−1·1	—	—	—
19·1	19·5	−0·4	0·74	0·77	−0·03
13·3	12·9	+0·4	0·57	0·56	+0·01
Mean difference		−0·2			−0·02
Standard error of the mean		±0·36			±0·02
		P† = 1 : 2 Not significant			P† = 1 : 2 Not significant

Vitamin A

Colorimetric method, Moore blue units per g. fat			Spectrophotometric method, mg. per 100 g. fat‡		
Kept	Original	Difference kept-original	Kept	Original	Difference kept-original
62	62	0	1·41	1·48	−0·07
60	58	−2	—	—	—
60	62	+2	1·18	1·42	−0·24
50	50	0	0·96	1·04	−0·08
Mean difference		0			−0·13
Standard error of the mean		±0·8			±0·055
		Not significant			P† = 1 : 10 Not significant

* These figures are calculated on the basis of $E^{1\%}_{1\,cm.} = 2200$ for pure carotene in $CHCl_3$ at 463 mμ[3].

† P = Probability that a difference at least as great as the observed difference would have arisen by random sampling from a homogeneous population.

‡ Based on the assumption that vitamin A itself has an intensity of absorption at 328 mμ $E^{1\%}_{1\,cm.} = 1600$ (in alcohol).

Summary

1. The vitamin A and carotene content of raw milk have been compared with that of milk from the same bulk after it had been commercially sterilized.

2. For this purpose the fat was obtained by ether extraction from eleven

samples of raw milk and from eleven corresponding samples of sterilized milk. As the sterilized milk had been previously homogenized it was not possible to churn it.

3. The vitamin A and carotene content was estimated by colorimetric (Lovibond tintometer) and by spectrophotometric tests.

4. The results showed that the effect of the heat treatment was negligible, neither method demonstrating a loss in vitamin A, while for carotene only the colorimetric method indicated a loss of about 2%.

5. The vitamin A and carotene contents of sterilized milk were not decreased after storage for several weeks in a cool dark place.

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174. THE EFFECT OF COMMERCIAL STERILIZATION ON THE NUTRITIVE VALUE OF MILK

IV. EFFECT ON THE VITAMIN B COMPLEX, ON VITAMIN B_1 AND ON VITAMIN B_2 (FLAVIN) OF MILK

By K. M. HENRY and S. K. KON

(With 3 Figures)

In previous work in this laboratory Kon(1) found that commercial pasteurization causes a certain loss of the vitamin B complex in milk. This loss could only be detected by the use of the faster-growing male rats, consequently in the present experiments only male rats were used.

Three series of experiments were carried out. The first was designed to determine the effect of sterilization on the vitamin B complex of milk. In the second series the effect on vitamin B_1 was studied, and the third dealt with the vitamin B_2 complex.

Experimental

In the three series of experiments the rats were prepared for the tests by the routine method employed in this laboratory. They were placed at weaning on a diet deficient in the vitamin B complex. Details of the composition of the diet will be found elsewhere(1). When the rats had become "run-out", i.e. after they had shown a loss in weight at two consecutive weighings within a week, the experimental feedings were started.

(I) *The vitamin B complex*

After having been "run-out" on the vitamin B complex deficient diet ten pairs of littermate male rats were allotted by toss of coin to either raw or sterilized milk. The rats were kept in cages in groups of three or four and were given 8 ml. of milk daily (with the exception of Sundays) in special individual feeding cages. The milk was consumed rapidly and quantitatively. In a number of cases, especially on sterilized milk, symptoms of beri-beri developed, the daily ration of milk was then raised to 12 ml. If the symptoms were severe the rats were placed in separate cages.

The growth curves of the rats are given in Fig. 1 and it will be seen that the growth of the rats on sterilized milk was markedly inferior to that of the rats receiving raw milk. Table I gives the individual figures for the gains in weight of the rats after 4 and 8 weeks on the experimental milks. It will be seen that after 4 weeks the gains in weight of the rats receiving raw milk were already markedly superior to those of the rats on sterilized milk. Four of the rats given

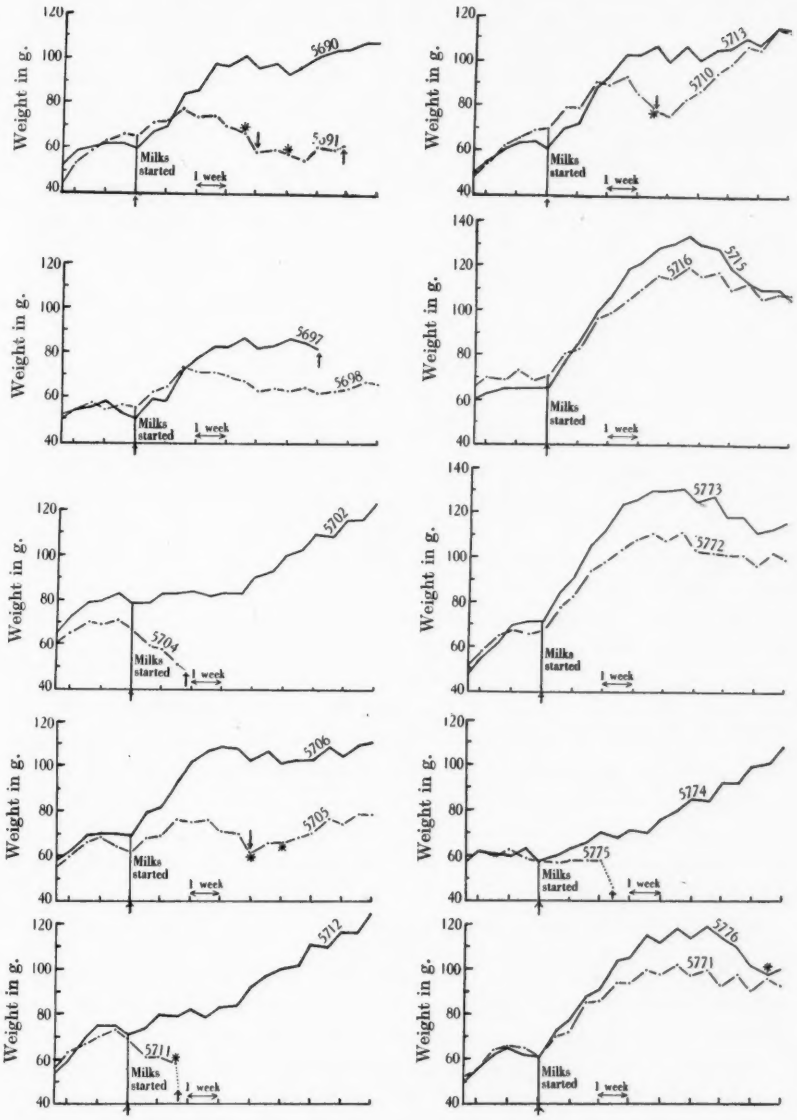

Fig. 1. The vitamin B complex content of raw and sterilized milk. Growth curves of rats receiving a diet deficient in the vitamin B complex and 8 ml. daily of either raw or sterilized milk.

—— Raw milk. ·—· Sterilized milk.

* Beri-beri symptoms. ↓ Milk raised to 12 ml. ↑ Dead.

Table I. *Gains in weight after 4 and 8 weeks of rats receiving raw or sterilized milk as the sole source of the vitamin B complex*

	Raw milk			Sterilized milk		
		Gain in wt. (g.)			Gain in wt. (g.)	
Litter no.	Rat no.	4 weeks	8 weeks	Rat no.	4 weeks	8 weeks
I	5690	36	48	5691	−6*	Dead
II	5697	32	Dead	5698	8	11
III	5702	11	45	5704	Dead	Dead
	5706	33	41	5705	0*	16*
IV	5712	21	54	5711	Dead†	Dead
	5713	39	54	5710	6*	44*
V	5715	64	40	5716	44	36
VI	5773	58	45	5772	40	32
	5774	18	50	5775	Dead	Dead
	5776	51	39*	5771	37	32

* Symptoms of beri-beri. Milk intake increased to 12 ml. daily in the course of this period.
† Symptoms of beri-beri.

the latter milk developed within that time marked symptoms of beri-beri. One of them (5711) died the next day. The three others (5691, 5705 and 5710) had their milk allowance raised to 12 ml. Two of them (5705 and 5710) recovered and grew slowly to the end of the experiment. The third one (5691) showed only a temporary improvement and death followed after recurrence of the symptoms.

One other rat (5704) became very spastic, but died without showing definite symptoms. Finally rat 5775 lost weight and died.

Against this there were no casualties within 4 weeks in the raw milk group, but within 8 weeks one rat in that group (5697) died or was killed by its cage-mates and another (5776) developed symptoms of beri-beri towards the end of the experiment, but recovered on having its milk increased to 12 ml.

The results show very clearly that sterilization of milk causes considerable destruction of some component or components of the vitamin B complex, and the incidence of convulsions in the sterilized milk group indicates that vitamin B_1 was markedly affected.

(II) *Vitamin B_1*

When rats for this and for the vitamin B_2 experiment became available it was found that the milk supply would cease in the course of 9 weeks. The rats were therefore placed on experiment before they were fully "run out".

Six pairs of littermate male rats were allotted by toss of coin to either raw or sterilized milk. 4 ml. of raw milk or 6 ml. of sterilized milk were given daily (Sundays excepted). A further six pairs of littermate male rats received either 8 ml. of raw milk or 12 ml. of sterilized milk daily. In addition to the milk each rat was given daily 2 ml. of a solution of autoclaved Marmite[1] equivalent to 0·63 g. of fresh Marmite.

[1] The Marmite was dissolved in twice its weight of water and was brought with 10% NaOH to *p*H 9. It was then autoclaved for 1 hr. at 120° C., cooled and brought to *p*H 5 with conc. HCl.

The growth curves of the rats are shown in Fig. 2. Individual figures for the gains in weight of the experimental animals at 4 and 8 weeks will be found in Table II. It can be seen both from Fig. 2 and from Table II that on the lower level of milk intake there was no difference in the rate of growth of the two groups of rats at the end of 4 weeks. After this two rats (5881 and 5890) on raw milk developed symptoms of beri-beri. When the milk intake was

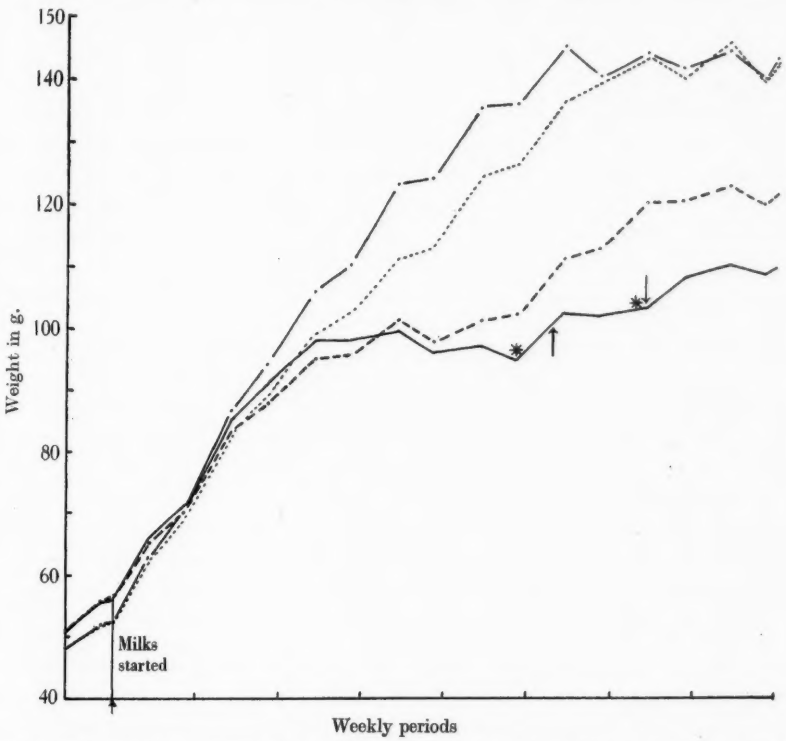

Fig. 2. The vitamin B_1 content of raw and sterilized milk. Composite growth curves (6 ♂♂ each) of rats receiving a diet deficient in vitamin B_1 and either 4 or 8 ml. daily of raw milk or 6 or 12 ml. daily of sterilized milk.

* Symptoms of beri-beri. ↑ Death.
↓ Milk increased to 6 ml. for one rat, thereafter rat not included in curve.
·—· 8 ml. raw milk. ----- 12 ml. sterilized milk.
·----· 6 ml. sterilized milk. ——— 4 ml. raw milk.

raised to 6 ml. daily one rat (5890), recovered, but the other (5881), died. At the end of 8 weeks better growth was shown by the rats receiving the sterilized milk. Of the rats receiving the higher levels of milk the raw milk ones showed better growth after 4 weeks than their littermates on sterilized milk; after 8 weeks, however, the gains of the two groups of rats were almost identical.

Table II. *Gains in weight after 4 and 8 weeks of rats receiving raw or sterilized milk as the sole source of vitamin* B_1

	Raw milk			Sterilized milk		
		Gain in wt. (g.)			Gain in wt. (g.)	
Litter no.	Rat no.	4 weeks	8 weeks	Rat no.	4 weeks	8 weeks
		4 ml. daily			6 ml. daily	
I	5881	22	Dead*	5882	28	60
II	5888	38	40	5887	42	47
	5890	29	24*†	5893	35	48
III	5898	62	69	5899	46	91
IV	5913	44	54	5912	40	55
V	5918	47	51	5920	59	87
	Average	40·3	53·5		41·7	64·7
		8 ml. daily			12 ml. daily	
V	5921	83	107	5922	80	120
VI	5931	62	88	5928	62	95
VII	5933	88	103	5934	67	101
	5936	74	81	5938	53	67
VIII	5950	65	69	5953	54	69
IX	5962	73	89	5961	67	88
	Average	74·2	89·5		63·8	90·0

* Symptoms of beri-beri.

† Milk increased to 6 ml. daily, this rat is therefore omitted from the average.

Coward *et al.* (2) have shown that, in vitamin B_1 tests on rats by the growth method, the increase in weight is proportional to the logarithm of the dose. We have therefore plotted the gains in weight of the rats on raw milk after 4 and 8 weeks against the logarithms of the doses (4 and 8 ml.) and obtained thus two straight lines. From these lines we calculated the number of millilitres of raw milk equivalent in growth response to the two doses of sterilized milk (6 and 12 ml.). The percentage destruction of vitamin B_1 in sterilized milk was then calculated from these values.

The results obtained by this method are shown in Table III.

Table III. *The vitamin* B_1 *content of sterilized milk expressed in raw milk equivalents. Percentage destruction of vitamin* B_1 *in sterilized milk*

Sterilized milk	Equivalent in raw milk (ml.)		% destruction of vitamin B_1	
ml.	4 weeks	8 weeks	4 weeks	8 weeks
6	4·15	5·01	31	17
12	6·52	8·00	46	33
	Average for both levels and periods		30	

In considering these figures it should be remembered that comparatively small groups of rats were used in this experiment. It can be seen that on both levels of milk intake the percentage destruction of vitamin B_1 as estimated by this method is less when the gains at 8 weeks are used than when the 4-week figures are employed. The significance of the 8-week comparison on the lower 4 ml. level of milk intake is somewhat diminished by the fact that, for comparative purposes, two of the rats receiving raw milk had to be discarded. The dose of vitamin B_1 contained in this amount of milk was on the borderline of protection, and two rats out of six developed acute symptoms of vitamin

B_1 deficiency. On the higher level of milk intake the rats grew satisfactorily for about 6 weeks, those on raw milk gaining more than those on sterilized. After that there was very little growth and the growth curves for the two groups became nearly identical, the sterilized having caught up with the raw group which had previously shown better growth.

It is evident from this experiment that sterilization has caused destruction of vitamin B_1. This destruction amounts probably to about 30% of the vitamin B_1 originally present in the milk.

(III) *Vitamin B_2 (flavin)*

The raw and sterilized milks were fed at two levels as in the experiment on vitamin B_1. In this experiment littermate rats received equal amounts of the two milks. 2 and 4 ml. of each milk were fed daily (Sundays excepted). In addition to the milk each rat received daily 0·1 ml. of a vitamin B_1 concentrate[1] equivalent to 5 I.U. of vitamin B_1. Six pairs of littermate male rats were placed on each level of milk, and two negative control rats received the daily dose of vitamin B_1 without the addition of milk.

Fig. 3 gives the growth curves for the various groups of rats, while individual gains at 4 and 8 weeks will be found in Table IV. The results of the statistical

Table IV. *Gains in weight after 4 and 8 weeks of rats receiving raw or sterilized milk as the source of vitamin B_2 (flavin)*

	Raw milk			Sterilized milk		
		Gain in wt. (g.)			Gain in wt. (g.)	
Litter no.	Rat no.	4 weeks	8 weeks	Rat no.	4 weeks	8 weeks
			2 ml. daily			
I	5884	50	Dead	5883	63	(80)*
	5885	47	74	5880	50	62
II	5889	42	75	5891	67	93
III	5896	35	56	5897	55	101
	5900	63	116	5901	44	84
IV	5914	54	104	5915	53	105
	Average	48·5	85·0		55·3	89·0
			4 ml. daily			
V	5919	70	114	5923	57	89
VI	5926	67	116	5927	63	94
	5930	60	100	5929	57	93
VII	5937	62	120	5935	69	109
VIII	5942	76	122	5943	73	125
IX	5952	58	98	5951	68	109
	Average	65·5	111·7		64·5	103·2
Negative controls:						
X	5963	35	64	—	—	—
	5964	30	48	—	—	—

* Omitted from the average.

[1] The concentrate was kindly given us by Mr A. L. Bacharach of Messrs Glaxo Laboratories, Ltd. According to him the concentrate contains no more than traces of flavin. It may, however, have contained other members of the vitamin B complex. The experiment was, therefore, in a strict sense rather an estimation of the effect of sterilization on lactoflavin than on the whole vitamin B_2 complex.

treatment of the differences in gain in weight on the two milks are shown in Table V.

The two negative control rats grew fairly steadily at a subnormal rate, but both had scaly feet at the end of the experiment and one of them had lost

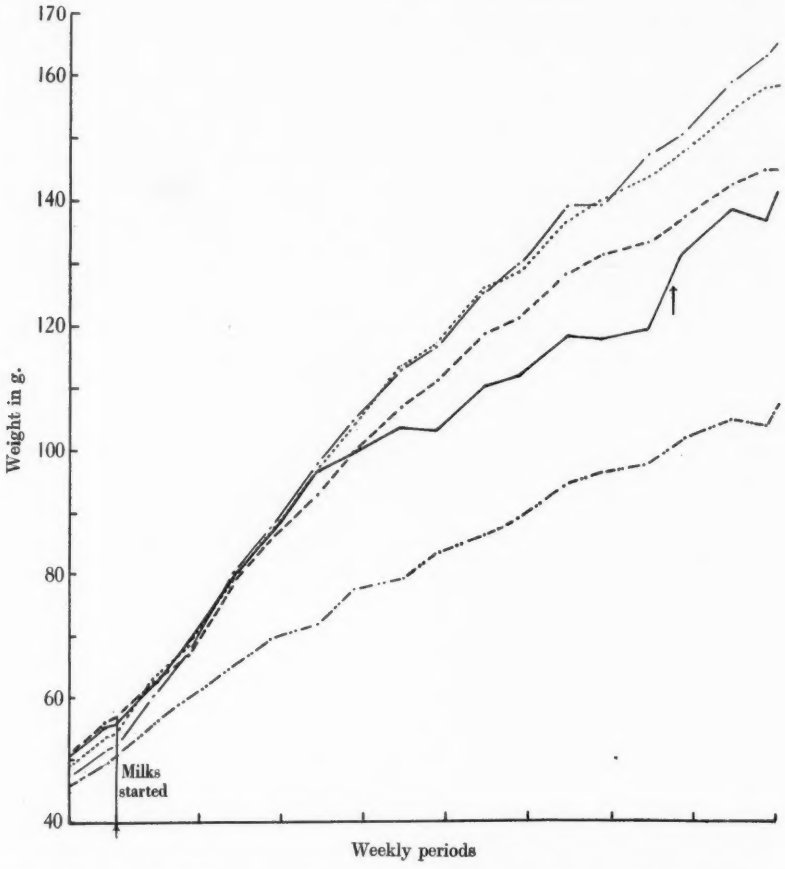

Fig. 3. The vitamin B_2 (flavin) content of raw and sterilized milk. Composite growth curves (6 ♂♂ each) of rats receiving a diet deficient in vitamin B_2 (flavin) and 2 or 4 ml. daily of either raw or sterilized milk.

↑ Death. ·—· 4 ml. raw milk. ----- 4 ml. sterilized milk.
·---· 2 ml. sterilized milk. —— 2 ml. raw milk. ·—··—· Negative controls.

most of its hair and its paws were badly cracked. One rat (5884) receiving raw milk died of pneumonia in the course of the experiment. The gain in weight of its littermate for the 8-week period is therefore not included in the average.

Table V. *Statistical significance of the differences in gains in weight of the rats receiving raw or sterilized milk as the source of vitamin B_2 (flavin)*

	Gain in g. on 2 ml. milk daily		Gain in g. on 4 ml. milk daily	
	4 weeks	8 weeks	4 weeks	8 weeks
Average for raw milk	48·5	85·0	65·5	111·7
Average for sterilized milk	55·3	89·0	64·5	103·2
Difference (raw-sterilized)	−6·8	−4·0	+1·0	+8·5
S.E.M.	±6·54	±13·11	±3·40	±5·70
P*	1 : 3	1 : 1	1 : 1	1 : 5
	Not significant	Not significant	Not significant	Not significant

* P = Probability that a difference at least as great as the observed difference would have arisen by random sampling from a homogeneous population.

Fig. 3 and Table IV show that there was little difference in the gains of weight of the rats receiving raw or sterilized milk at either the lower or at the higher level. At the lower level of intake the rats receiving sterilized milk showed slightly better growth both at 4 and 8 weeks than their littermates on raw milk, while the reverse was true on the higher level of intake. Table V shows that these differences are without statistical significance.

It appears therefore that sterilization had no adverse effect on the vitamin B_2 (flavin) of milk.

SUMMARY

1. The effect of commercial sterilization on the vitamin B complex, vitamin B_1 and vitamin B_2 (flavin) of milk has been investigated in experiments on rats using milk from the same bulk before and after heat treatment.

2. No quantitative comparisons were carried out regarding the vitamin B complex, but the tests showed that a considerable destruction of some component or components of it had been caused by the heat treatment and that vitamin B_1 was, most probably, one of them.

3. Separate tests showed that about one-third of the vitamin B_1 originally present in milk was destroyed by sterilization.

4. Sterilization appeared to have no adverse effect on vitamin B_2 (flavin).

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175. THE PRECIPITATION OF THE PROTEINS IN MILK

I. CASEIN II. TOTAL PROTEINS III. GLOBULIN IV. ALBUMIN AND PROTEOSE-PEPTONE

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(With 1 Figure)

In a previous paper(1) it was shown that secondary proteins of a proteose-peptone nature are present, with albumin and globulin, in the soluble protein fraction of normal cow's milk. In the present paper improved methods are described for the precipitation and determination of all the milk proteins—casein, albumin, globulin and the proteose-peptone substances. The methods should prove of value on account of the importance of a detailed and accurate knowledge of the protein distribution in studies of milk composition, and of the physiology of normal and abnormal milk secretion.

I. THE PRECIPITATION OF CASEIN

Casein constitutes over 80% of the total protein of normal milk, globulin only 3–4%. The failure to precipitate an extremely small proportion of the casein as such causes a large percentage error in the globulin figure, for, in protein distribution determinations, unprecipitated casein is recovered subsequently as globulin.

Casein is a protein typically insoluble at its isoelectric point, a *p*H of 4·6–4·7. Moir(2) has discussed the advantages, over the many other methods that have been proposed, of the quantitative precipitation of casein at its isoelectric point by means of an acid reagent. He proposed the addition of 1·5 ml. of 10% acetic acid, and, 20 min. later, 4·5 ml. of *N*/4 sodium acetate, to 10 ml. of milk diluted with about 50 ml. of water; the casein precipitate being filtered off, washed, and digested for the determination of its nitrogen content by the Kjeldahl method. Moir showed that his method gives results for casein which are 1–2% higher than results obtained by the much-used method of the Association of Official Agricultural Chemists—a method employing the acetic acid alone, and precipitating casein at a *p*H far below its isoelectric point.

The writer confirmed Moir's observation, but was not satisfied that the precipitation of casein was complete, as an examination of the data on which his method was based revealed that the effect of less than 1·5 ml. of acetic acid per 10 ml. of milk had not been explored. The data, in fact, served to indicate the amount of sodium acetate which produces the maximum casein precipita-

tion with 1·5 ml. of 10% acetic acid. In some preliminary experiments, the writer found that, using no sodium acetate, only about 0·7 ml. of acid was required to bring 10 ml. of milk to the isoelectric point of casein. The addition of acid in marked excess would disperse some casein as casein acetate, and it was unlikely that the subsequent addition of sodium acetate to raise the *p*H to the isoelectric point would result, in a reasonably short time, in the recovery as insoluble casein of all of the casein so dispersed.

The advantage of the acetate as a buffer with milk samples of varying casein content and acidity was appreciated, but it was considered likely that the use of less than 1·5 ml. of acid would increase the casein precipitation. To settle this point, the effect of varying the amounts of both acetic acid and sodium acetate was investigated, and a technique worked out for the determination of casein which dispensed with the tedious washing of precipitates, shortened the time of working, and increased the accuracy of the result.

EXPERIMENTAL

The percentages of casein precipitated from a number of milk samples by different amounts of acetic acid and sodium acetate were determined in the following manner. 10 ml. of the sample were weighed in a 100 ml. graduated flask, and about 80 ml. of water at 40° C. added, followed by the required volume of 10% acetic acid. After 10–15 min. the required volume of *N* sodium acetate was added, and the flask allowed to cool. The volume was then made up to 100 ml. with water, and the contents of the flask well mixed and filtered on a dry paper into a dry flask. The nitrogen content of the filtrate was determined by digesting 20 ml. in a 200 ml. Kjeldahl flask with 5 ml. of sulphuric acid, 2 g. of potassium sulphate, 0·2 g. of copper sulphate, and 2 drops of selenium oxychloride. The ammonia was distilled into 15 ml. of *N*/50 sulphuric acid, and the excess acid titrated with *N*/50 sodium hydroxide. The percentage of total nitrogen in the milk sample was determined by digesting 1 ml. in a similar way, and distilling the ammonia into 25 ml. of *N*/50 acid. This semi-micro method is described in detail in the next paper, as applied to the determination of the full nitrogen distribution in milk. The percentage of casein nitrogen was obtained as the difference between the total and casein-free filtrate nitrogen. It was necessary to reduce the apparent nitrogen content of this filtrate slightly to correct for the volume occupied in the 100 ml. flask by the precipitate of casein and fat from 100 ml. of milk. The average factor to be applied was 0·995, as shown fully on p. 43. The *p*H values of the casein free filtrates were measured with the quinhydrone electrode.

RESULTS

All figures are the mean of duplicates which agreed closely. The results of the first series are given in Table I A. It will be seen that with a constant volume of 1 ml. of *N* sodium acetate the maximum precipitation took place

with the smallest volume of acetic acid used, namely 1·0 ml., and gave a casein nitrogen percentage of 0·4233. This was 2·4 and 1·0% greater than the figures given by the quantities of reagents used in the A.O.A.C. and Moir methods respectively; and therefore more extensive series were carried out on other milk samples, some of which are recorded in Tables I B and II. Details of all the experiments made are not presented, as they lead to the same conclusions as the data of these tables.

Table I. *Precipitation of casein from milk samples A and B*

10% acetic acid ml.	*N* sodium acetate ml.	*p*H of filtrate	Percentage of casein nitrogen
	Milk A		
1·00	1·0	4·67	0·4233
1·25	1·0	4·61	0·4225
1·50	1·0	4·67	0·4193
1·75	1·0	4·40	0·4175
2·00	1·0	4·36	0·4150
1·50	—	3·98	0·4135
1·50	2·0	4·71	0·4190
1·50	3·0	4·88	0·4180
	Milk B		
0·75	0·5	4·73	0·3805
1·00	0·5	4·56	0·3800
1·00	1·0	4·66	0·3805
1·00	1·5	4·84	0·3788
1·50	0·5	4·42	0·3734
1·50	1·0	4·49	0·3752
1·50	1·5	4·56	0·3773
2·00	3·0	4·68	0·3734

Table II. *Precipitation of casein from milk sample C*

10% acetic acid ml.	*N* sodium acetate ml.	*p*H of filtrate	Percentage of non-casein nitrogen	Percentage of casein nitrogen
0·75	—	4·56	0·1234	0·4151
,,	0·25	4·68	0·1222	0·4163
,,	0·50	4·80	0·1212	**0·4173**
,,	1·00	4·96	0·1218	0·4167
1·00	—	4·37	0·1260	0·4125
,,	0·25	4·49	0·1244	0·4141
,,	0·50	4·59	0·1222	0·4163
,,	1·00	4·75	0·1218	**0·4167**
,,	1·50	4·89	0·1260	0·4125
,,	2·00	4·96	0·1266	0·4119
1·50	—	4·14	0·1370	0·4015
,,	0·50	4·39	0·1282	0·4103
,,	1·00	4·51	0·1267	0·4118
,,	1·50	4·63	0·1255	**0·4130**
,,	2·00	4·75	0·1255	**0·4130**
,,	3·00	4·84	0·1267	0·4118
,,	4·00	4·92	0·1277	0·4108
2·00	—	4·00	0·1464	0·3921
,,	1·00	4·35	0·1370	0·4015
,,	2·00	4·52	0·1272	**0·4113**
,,	3·00	4·70	0·1290	0·4095

In some casein determinations acetic acid was added to bring the milk samples to the point of maximum precipitation directly (no addition of sodium acetate), and it was found that 0·65–0·70 ml. of 10% acid per 10 ml. of milk

gave the best results. The casein percentage, however, was not significantly different from that obtained on the same samples with 0·75 ml. of acid plus 0·50 ml. of acetate, or 1·0 ml. of acid plus 1·0 ml. of acetate, and it was observed that the time taken for the precipitation was increased. It took much longer for the casein particles to settle and leave a clear supernatant liquid.

DISCUSSION OF RESULTS

Maximum casein precipitation

The results in Table I B show maximum precipitation with 0·75 ml. of acid plus 0·5 ml. of acetate, and with 1·0 ml. of acid plus 1·0 ml. of acetate. The casein nitrogen figure 0·3805 is an increase of 1·4% over the figure 0·3752 obtained with the amounts of precipitants proposed by Moir. Table II is in agreement for milk sample C, maximum precipitation being 3·8 and 1·2% greater than by the A.O.A.C. and Moir methods respectively. The above data permit of a number of conclusions concerning the precipitation of casein by the acetic acid-sodium acetate buffer, and fix the conditions for maximum precipitation.

(1) The greater the excess of acid added initially, the smaller the amount of casein recovered, notwithstanding the addition, for each volume of acid, of a corresponding optimum volume of sodium acetate.

(2) When there is no acid in excess initially, the rate of precipitation of free casein is very slow, and the amount of casein subsequently recovered is the same as when a slight excess of acid (0·75–1·00 ml.) is followed by a suitable amount of acetate. A slight excess of acid initially is therefore an advantage in that it increases the rate of precipitation, and ensures that with milk samples of high casein content there is no unchanged dispersed calcium caseinate. The small amount of acetate rendered necessary is a valuable buffer, and makes it possible to use the same amounts of acid and acetate for milk samples with widely differing casein contents and acidity. (The addition to the milk of acetic acid and sodium acetate at the same time, in the form of a buffer giving a suitable final *p*H for the mixture, always results, as Moir showed, in incomplete casein precipitation.)

(3) The addition of a marked excess of acid initially—more than 1·0 ml. per 10 ml. of milk—takes the *p*H far below the isoelectric point of casein and disperses a portion of the casein as casein acetate. The addition of acetate afterwards, although raising the *p*H suitably, does not cause precipitation of all of that dispersed casein, as the change from casein acetate to free casein is slow.

(4) With increasing amounts of acetic acid, and hence of sodium acetate, the *p*H of maximum precipitation decreases. The results of all the experiments, summarized in Table III, accord with the observations of Sørensen & Sladek (3) who showed that the *p*H of minimum solubility of casein varied with the salt content of the casein solution.

Table III

10% acetic acid ml.	*N* sodium acetate ml.	*p*H of maximum casein precipitation
0·75	0·5	4·75–4·80
1·00	1·0	4·65–4·75
1·50	1·5	4·60–4·65
2·00	2·0	4·55–4·60

(5) The maximum precipitation of casein from milk is attained by adding, per 10 ml. of the sample, 1·0 ml. of 10% acetic acid solution, and, 10–15 min. later, 1·0 ml. of *N* sodium acetate solution. The 10 ml. of milk is first diluted with distilled water at 40° C.; the writer has found that the amount of casein precipitated is the same whether the milk be diluted to 50 or 100 ml.

The experimental technique

The method by which the casein nitrogen was determined as the difference between the total nitrogen and the non-casein nitrogen was found to offer several advantages over the usual method of determining the casein nitrogen direct.

(i) Very small differences in the amounts of casein removed are measurable —see Table II, column 4.

(ii) Washing and transfer of the casein precipitate are avoided. These processes take time, and there is always the risk, towards the end of the washing, of peptizing small amounts of casein.

(iii) The lengthy digestion of a filter paper and a protein precipitate containing fat are avoided. The digestion of the casein-free filtrate, and the conversion of its nitrogen to ammonia, are much more rapid, and duplicate determinations agree much more closely.

These advantages in greater ease of working, speed and accuracy should commend themselves to the analyst. Elsdon (4) accepted Moir's method for the determination of casein as the best then available, but wrote "there is a somewhat urgent need for a standard process for the determination of casein which will be both rapid (all processes which involve the washing of precipitated casein are tedious and likely to give erroneous results) and accurate". The method arrived at in this paper should meet these requirements. It is not often that one wants the percentage of casein nitrogen in a milk sample without the percentage of total nitrogen. If such be the case, in order to limit the nitrogen determinations, the casein precipitate obtained as above may be washed and digested. Then the improved accuracy of the figure is obtained, but the advantages of ease and speed are lost.

The Kjeldahl procedure employed for the above work was on a rapid semi-micro scale. The usual macro-Kjeldahl digestion and distillation methods, although slower, may equally well be applied, using 5 ml. of milk for total nitrogen and 50 ml. of the casein-free filtrate for non-casein nitrogen.

The casein nitrogen percentages, as used throughout, may be converted into the corresponding casein percentages by multiplying by 6·38.

II. THE PRECIPITATION OF THE TOTAL PROTEINS

About 5% of the total nitrogen of normal milk is in the form of amino-acids, urea, creatine, creatinine, uric acid, and ammonia. It is referred to as non-protein nitrogen to distinguish it from the nitrogen in the form of casein, lactalbumin, lactoglobulin, and secondary proteins of a proteose-peptone nature. The blood of the cow contains the same residual nitrogenous substances, and as a result much attention has been given to the non-protein nitrogen content of milk in the diagnosis of disturbed secretion, where the trouble may be due to bacterial invasion of the udder (mastitis, tuberculosis), the end of lactation, or the production of milk with a low content of solids-not-fat (5, 6, 7, 8).

Many reagents have been employed for the removal of the total proteins from milk, including tannic acid, phosphotungstic acid, tungstic acid, uranyl acetate, and trichloroacetic acid. These reagents all give different values for the protein and non-protein nitrogen. Munchberg (5) compared several precipitants and found that phosphotungstic acid, sodium tungstate-trichloroacetic acid mixture, and uranyl acetate with normal milk gave non-protein nitrogen percentages of 0·028, 0·024 and 0·023 respectively. Kieferle & Gloetzl (9) found with 5% trichloroacetic acid, phosphotungstic acid, and sodium tungstate-trichloroacetic acid mixture, 0·0312, 0·0286 and 0·0234% non-protein nitrogen respectively. Sanders (10) gave as average values tungstic acid 0·0278 and 8% trichloroacetic acid 0·0322% non-protein nitrogen.

With the exception of trichloroacetic acid, the reagents mentioned precipitate some fraction of the non-protein nitrogen—e.g. phosphotungstic acid precipitates diamino-acids—and give low non-protein nitrogen and high total protein nitrogen figures. Also they yield precipitates which are bulky, gelatinous, slow to filter, and difficult to free from adsorbed and occluded substances. Trichloroacetic has several desirable properties; it is used in aqueous solution, gives a fibrous precipitate which filters rapidly, furnishes a large volume of filtrate, and does not interfere with subsequent digestion for nitrogen determination. Further, it does not precipitate amino-acids, but in sufficiently strong solution will precipitate secondary proteins.

Moir (11) proposed tentatively a method for the precipitation of the total proteins from milk with hot 4% trichloroacetic acid, adding, to 5 ml. of milk, 35 ml. of water and 10 ml. of 20% acid. The mixture was maintained at 70° C. for 30 min., allowed to cool, and filtered. The precipitate was washed and its nitrogen content determined, the non-protein nitrogen being obtained as the difference from the total nitrogen of the milk determined separately. Sanders (10) recommended for the precipitation of the milk proteins the addition of 4 volumes of 10% trichloroacetic acid to one volume of milk, at room temperature. The precipitate was filtered on a dry paper and the non-protein nitrogen content of an aliquot part of the filtrate determined directly. This method appealed to the writer as it combines ease and speed, with the possi-

bility of greater accuracy, in the very small figure for the non-protein nitrogen content of the milk. The total protein nitrogen may then be determined as the difference from the total nitrogen, while the transfer, washing, and digestion of the protein plus fat precipitate are avoided.

The literature was found to contain ample evidence of the need for high concentrations of trichloroacetic acid to ensure complete precipitation of proteins and proteose-peptone substances, and for a low temperature to avoid proteolysis by the acid. The methods of Moir and Sanders described above were therefore compared, and were followed by an investigation of the precipitating action of trichloroacetic acid in concentrations up to 18% with the object of selecting the most satisfactory concentration for regular use.

Experimental

1 (*a*). 10 ml. of milk were taken in a number of 100 ml. graduated flasks and 70 ml. of water and 20 ml. of 20% trichloroacetic acid added. The flasks were maintained at various temperatures (40–100° C.) for various periods by immersion in thermostats, and then cooled and filtered on dry papers. The nitrogen content of 40 ml. of each of the filtrates was determined by the Kjeldahl method, the ammonia being distilled into $N/70$ sulphuric acid. This procedure therefore precipitated the proteins with hot 4% acid as in Moir's method, but the technique of Sanders was used.

1 (*b*). 10 ml. of the same milk were taken in a 50 ml. graduated flask, 40 ml. of 10% acid added, and the whole well mixed. The proteins were allowed to settle, and the contents of the flask were filtered and treated as in 1 (*a*). The nitrogen content of 20 ml. of the filtered was determined, and gave the non-protein nitrogen content of the milk by Sanders' method.

The essential data from a typical set of results are given in Table IV.

2. 10 ml. of milk were taken in 50 and 100 ml. flasks and trichloroacetic acid solutions added up to the marks. The acid solutions were of such strengths that final concentrations of from 6 to 18% were obtained in the milk-acid mixtures, e.g. 12% acid was produced in a 50 ml. flask by filling up with 15%. The mixtures were treated as in 1 (*b*), 40 and 20 ml. of the filtrates were digested from the 100 and 50 ml. flasks respectively. A typical set of results is given in Table V.

Results

Table IV. *The precipitation of total proteins with trichloroacetic acid*

Concentration of acid %	Heat treatment of milk-acid mixture	mg. non-protein nitrogen per 100 ml. of milk
8	None	30·5
4	15 min. at 45° C.	31·4
4	5 min. at 50° C.	31·4
4	30 min. at 70° C.	34·2
4	30 min. at 100° C.	56·2

Table V. *The precipitation of total proteins with trichloroacetic acid at room temperature*

Milk sample no.	Concentration of acid %	mg. non-protein nitrogen per 100 ml. of milk	Milk sample no.	Concentration of acid %	mg. non-protein nitrogen per 100 ml. of milk
I. 10 ml. milk diluted to 100 ml.	8	32·4	III. 10 ml. milk diluted to 50 ml.	6	32·5*
	10	31·6		8	31·2*
	12	31·4		10	30·3
	14	31·2		12	29·8
	16	31·6*		14	29·6
	18	32·8*		16	29·6
II. 10 ml. milk diluted to 100 ml.	10	33·3	IV. 10 ml. milk diluted to 50 ml.	10	34·0
	12	33·0		12	33·2
	14	32·7		14	33·0
	16	33·0*		16	33·0

* Filtrate cloudy

DISCUSSION OF RESULTS

1. The data of Table IV show that a concentration of 4% trichloroacetic acid in the milk-acid mixture is not satisfactory. Heating at 70° C. for 30 min. caused hydrolysis of protein and high non-protein nitrogen values. The minimum non-protein nitrogen value with 4% acid was got by heating at 45° C. for 15 min. or at 50° C. for 5 min. Less vigorous heating produced a smaller precipitate, and more vigorous heating caused proteolysis. The method recommended by Sanders was much easier to carry out (8% acid at room temperature), and resulted in a greater removal of proteins.

Moir chose 4% trichloroacetic acid for the precipitation of milk proteins "as lower concentrations sometimes gave low results, while higher concentrations were avoided because of the work of Hiller & Van Slyke which indicated that heating in the presence of strengths of 5% and over was liable to cause hydrolysis". Actually, Hiller & Van Slyke(12) studied the action of several precipitants on the whole proteins and protein intermediate products of blood and Witte's peptone. Their results are not directly applicable to milk wherein the nature and relative amounts of the nitrogenous substances are different. Also they did not heat the mixture of proteins and trichloroacetic acid to promote precipitation. They found that 5% acid at room temperature precipitated only whole proteins, and 10% precipitated also proteoses and peptones and gave nearly as great a precipitation as tungstic acid. The hydrolysis which was observed with 5% acid took place after the precipitate had been filtered off, in fact when the filtrate containing protein intermediate products was being boiled to decompose trichloroacetic acid prior to the determination of amino-nitrogen.

2. The data of Table V show that a final concentration of acid greater than 8% is necessary for the maximum precipitation of proteins. The figures for milk samples I and II, where 10 ml. of milk were diluted to 100 ml., show

maximum precipitation at 14%, and for samples III and IV, where 10 ml. of milk were diluted to 50 ml., show maximum precipitation at both 14 and 16%. It will be seen that the very high acid concentrations increase the non-protein nitrogen by hydrolysis to an almost negligible extent. The milk-acid mixtures were normally filtered after 15–30 min., but the mixture from milk sample III, with acid at 14%, was also kept at room temperature for 24 hr., during which time the precipitate was repeatedly mixed with the acid. It was then found that the non-protein nitrogen had increased from 29·6 to 30·6 mg. % only.

There is such a minute change in the non-protein nitrogen figures with increase in concentration of trichloroacetic acid above 10–12% that for routine determinations a final concentration of 12% is recommended, 1 volume of milk being diluted to 5 volumes, e.g. dilute 10 ml. of milk to 50 ml. with 15% trichloroacetic acid. The precipitate may be filtered off, washed with dilute trichloroacetic acid, and digested by the Kjeldahl method, but it is preferable, as described earlier, to avoid transfer and washing of the precipitate by determining the non-protein nitrogen content and subtracting it from the total nitrogen content to get the total protein nitrogen. For details see pp. 42–43.

III. THE PRECIPITATION OF GLOBULIN

No simple method has been suggested for the determination of globulin. Moir(13) recommended measuring the globulin nitrogen as the difference between the nitrogen contents of (*a*) the casein precipitate, and (*b*) the casein plus globulin precipitate which was obtained by the addition, to 10 ml. of milk neutralized to phenolphthalein, of 90 ml. of saturated magnesium sulphate solution and sufficient solid sulphate to saturate the neutralized milk. Peskett & Folley(14) have criticized this method as it failed to accomplish a satisfactory separation of some artificially added blood serum proteins from casein. They considered the globulin figures open to doubt. The writer considers that the method may be improved for the following reasons:

(1) The precipitate of casein, globulin, and fat is heavy, gelatinous, and extremely slow to filter. Also it is impossible to wash it free of the unprecipitated nitrogenous substances by flooding the filter paper with saturated magnesium sulphate solution. The degree of dilution of the milk is not sufficient for complete precipitation.

(2) "Neutralization" of the milk with sodium hydroxide, using phenolphthalein as indicator, as directed gives a solution of *p*H at least 8·5. At this *p*H some albumin will be precipitated with the globulin.

(3) The globulin content of normal milk is so low that it cannot be determined accurately as the small difference between two relatively large figures both liable to an experimental error.

The method which has been described in Part I for the precipitation of casein, i.e. filtering a milk-acetic acid-sodium acetate mixture of known volume on a dry filter paper, has made it possible to work out a method for

the determination of globulin which is free from the above objections. It is as follows: to 10 ml. of milk in a 100 ml. graduated flask add water, acetic acid and sodium acetate as described in Part I for the complete precipitation of casein, and filter on a dry paper. Pipette an aliquot of the casein-free filtrate into a beaker, add a few drops of bromcresol purple or bromthymol blue solution as indicator, and sodium hydroxide solution until the *p*H is within the range 6·8–7·2. Then add powdered crystalline magnesium sulphate, to saturate the solution, at the rate of 9 g. per 10 ml. of solution. The sulphate will dissolve readily if the solution be stirred and warmed to 25–30° C. After some hours filter off the precipitate of globulin, wash it with saturated magnesium sulphate, and determine its nitrogen content.

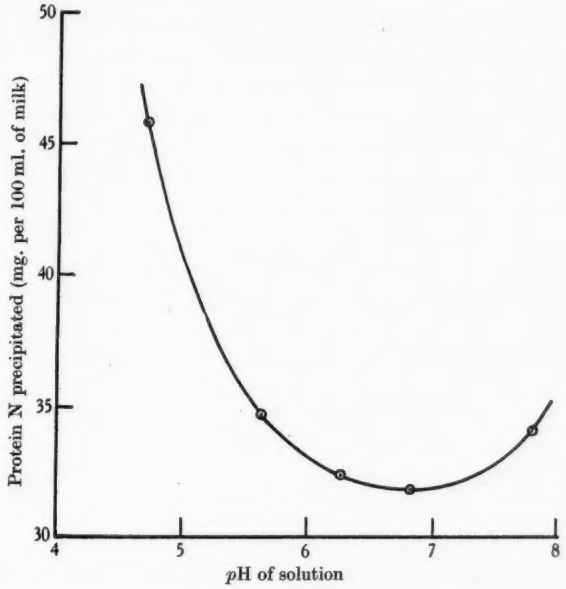

Fig. 1. Effect of *p*H on separation of globulin from albumin.

The degree of accuracy with which the *p*H of the casein-free filtrate must be controlled was found by adding varying amounts of sodium hydroxide to portions of it, and measuring the *p*H electrometrically before saturating with sulphate. The results, recorded in Table VI (and shown graphically in Fig. 1),

Table VI. *Effect of p*H *on the separation of globulin from albumin*

*p*H of the solution	"Globulin" nitrogen, mg. per 100 ml. of milk
4·70	45·8
5·63	34·7
6·26	32·3
6·81	31·8
7·78	33·8

indicate that to avoid the precipitation of some albumin along with the globulin the *p*H must first be adjusted to near the neutral point, within *p*H 6·8–7·2.

IV. THE PRECIPITATION OF ALBUMIN AND PROTEOSE-PEPTONES

The soluble protein other than globulin has usually been considered to consist entirely of albumin, but Rowland (1), by precipitation experiments, has produced evidence of the presence of proteose-peptone substances in milk; and Pedersen (15), by ultra-centrifugal studies of milk serum, has shown the presence of three different proteins including a protein of rather low molecular weight.

Direct methods for the precipitation of albumin and proteose-peptone free from casein and globulin entail the treatment of saturated magnesium sulphate solution. The magnesium sulphate solution obtained after the precipitation of casein and globulin may be acidified to precipitate albumin alone, and treated with a protein precipitant such as trichloroacetic acid to remove albumin plus proteose-peptone, but these procedures are unsatisfactory for quantitative work. Albumin plus proteose-peptones are readily determined as the difference between the soluble protein and the globulin.

The separation of the albumin from the proteose-peptone substances is accomplished by precipitating the true albumin and the globulin in one of the following ways (1): (*a*) by adjusting the *p*H of the casein-free filtrate to 4·75, and boiling to denature and coagulate them; (*b*) by heating the milk at 95° C. for 10–20 min. and coprecipitating them in the denatured form along with casein at *p*H 4·7—thus leaving the proteose-peptone substances in solution. These substances can then be precipitated alone by adding trichloroacetic acid to give a final concentration of 8%.

SUMMARY

Improved methods have been evolved for the separation of the total protein, casein, albumin, globulin, and proteose-peptone substances of milk.

I. The use of acetic acid-sodium acetate solutions for the precipitation of casein is elucidated, and it is shown that the maximum precipitation of casein is rapidly effected from milk samples of varying casein content by the addition to 10 ml. of milk of about 80 ml. of water at 40° C. and 1·0 ml. of 10% acetic acid solution, followed, after 10 min., by 1·0 ml. of *N* sodium acetate solution.

This maximum precipitation was found to be 1·0–1·4% greater than by Moir's method, and 2·4–3·8% greater than by the method of the Association of Official Agricultural Chemists.

A procedure is suggested for the determination of casein which avoids the tedious transfer and washing of the precipitate, and gives enhanced ease, accuracy and speed of working.

II. The advantages of trichloroacetic acid for the precipitation of proteins in determinations of the total protein and the non-protein nitrogenous substances of milk are discussed.

The trichloroacetic acid methods at present in use are shown not to give complete precipitation, and for this a rapid method employing, at room temperature, a final concentration of 12% acid in the milk-acid mixture is recommended.

III. An accurate method for the precipitation of globulin uncontaminated with either albumin or casein is described.

IV. Methods are given for the precipitation and separation of the albumin and proteose-peptone substances.

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176. THE DETERMINATION OF THE NITROGEN DISTRIBUTION IN MILK

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Utilizing the methods of separation described in the previous paper(1), a semi-micro Kjeldahl method is given in detail for the determination of the total, casein, albumin, globulin, proteose-peptone, and non-protein nitrogen in milk. For the accurate determination of the small amounts of nitrogen in some of these fractions a normal macro Kjeldahl procedure is impracticable. A semi-micro procedure is suitable, and has advantages in economy of materials, speed, and ease of working. The method to be described has been used, with very satisfactory results, for the determination of the nitrogen distribution in a large number of samples of normal and abnormal milk.

Analytical procedure

I. *Total nitrogen*

Into a weighed 100 ml. graduated flask pipette 5 ml. of the milk, weigh, and dilute to the mark with water. Pipette 20 ml. of the diluted milk (=1 ml. of the original milk) into a 200 ml. Kjeldahl flask, and add 5 ml. of nitrogen-free sulphuric acid, 2 g. of potassium sulphate, 0·2 g. (approx.) of copper sulphate, and 2 drops of selenium oxychloride. Digest the contents of the flask by heating over a small flame, continuing the heating for 15 min. after the contents become clear. Allow to cool, add 50–60 ml. of water and excess (15 ml.) of 50% sodium hydroxide solution.[1] By steam distillation liberate the ammonia direct from the Kjeldahl flask, through a small bulb trap and condenser, into excess, normally 25 ml., of *N*/50 sulphuric acid to which 2–3 drops of 0·1% methyl red solution have been added. The condenser should have a plain vertical central tube made of Pyrex or Jena glass or of silver. The ammonia is completely removed in 10–15 min. Boil the distillate to remove carbon dioxide, cool, and titrate the excess of acid with carbonate-free *N*/50 sodium hydroxide from a 10 ml. burette reading to 0·02 ml.

II. *Non-casein nitrogen*

Into a 100 ml. graduated flask pipette and weigh 10 ml. of the milk. Add 70–80 ml. of water at 40° C. and 1·0 ml. of 10% acetic acid solution. Mix, and after 10 min. add 1·0 ml. of *N* sodium acetate solution and mix again. Allow

[1] Solution of 500 g. of sodium hydroxide, 500 ml. of water, and 125 g. of sodium thiosulphate crystals to prevent the formation of ammoniacal complexes with copper in alkaline solution.

the contents of the flask to cool, make up to the 100 ml. mark with water, mix, and stand for the precipitate of casein and fat to settle. Filter on a dry pleated No. 40, 11 cm. Whatman paper into a dry flask. Pipette 20 ml. of the filtrate into a 200 ml. Kjeldahl flask, and continue the digestion and distillation as in I, using 15 ml. of $N/50$ acid to receive the ammonia.

The non-casein nitrogen content of the milk found in this way has to be reduced slightly to correct for the volume occupied in the 100 ml. flask by the precipitate of casein and fat. This volume has been ascertained for a number of milk samples of varying fat and casein contents by a comparison of the nitrogen contents of the casein-free filtrates obtained in the following two ways:

(i) 10 ml. of milk, 1 ml. of 10% acetic acid, and 1 ml. of N sodium acetate made up to 100 ml. with water and filtered on a dry paper. The nitrogen content of 40 ml. of the filtrate was found.

(ii) 10 ml. of milk precipitated as in (i) and the precipitate well washed with dilute buffer solution to give a total filtrate of 250 ml. The nitrogen content of 100 ml. of the filtrate was found.

The volume of the precipitate from 10 ml. of average milk (3·7% fat, 2·6% casein) is 0·5 ml. The factor for the correction of the non-casein nitrogen is therefore 0·995. For rich milk samples (5·0% fat, 3·0% casein) the volume of the precipitate is 0·8 ml., and the correction factor 0·992. The volume of the precipitate may be calculated as $0{\cdot}11\,F + 0{\cdot}07\,C$, where F and C are the percentages of fat and casein respectively, but it is obvious that the use of the factor 0·995 in all cases introduces an extremely small error into the non-casein nitrogen figure, and a negligible error into the final casein nitrogen figure.

III. *Non-protein nitrogen*

Into a 50 ml. graduated flask pipette and weigh 10 ml. of the milk. Dilute to the mark with 15% trichloroacetic acid solution and mix immediately. When the precipitate has settled, leaving a clear supernatant liquid, filter on a dry pleated No. 40, 9 cm. Whatman paper into a dry flask. Pipette 20 ml. of the filtrate into a 200 ml. Kjeldahl flask, and continue the digestion and distillation as in I, using 10 ml. of $N/50$ acid to receive the ammonia.

IV. *Proteose-peptone plus non-protein nitrogen*

Method (1).

Pipette 25 ml. of the casein-free filtrate from II above into a 50 ml. beaker and add 0·25 ml. of $N/10$ sodium hydroxide solution to raise the pH to 4·75–4·80. Boil over a small flame for 5–10 min., and cool by standing the beaker in cold water. Transfer the contents to a 25 ml. graduated flask, and make up to the mark with water used to rinse the beaker. Mix and filter on a dry No. 40, 7 cm. Whatman paper into a dry flask. Pipette 20 ml. of the filtrate into a 200 ml. Kjeldahl flask, and continue the digestion and distillation as in I, using 10 ml. of $N/50$ acid to receive the ammonia.

If preferred, the precipitate of albumin and globulin may be filtered off, washed with water, and digested directly for the determination of its nitrogen content, in which case the proteose-peptone plus non-protein nitrogen is found by subtracting this direct albumin plus globulin figure from the non-casein nitrogen figure given by II above.

As stated in a previous paper(2), it is impossible to remove the last traces of albumin and globulin from the casein-free filtrate by boiling at *p*H 4·75. An amount of albumin and globulin nitrogen very close to 0·6 mg. per 100 ml. of the casein-free filtrate will remain with the proteose-peptone plus non-protein nitrogen, and should be allowed for.

Method 2.

As an alternative to the heat-coagulation of the casein-free filtrate, the milk may be heated at 95° C., for 10–20 min. and the denatured albumin and globulin precipitated at the same time as the casein by the method of II above(2). The filtrate then contains only proteose-peptone and non-protein nitrogen, and there is no correction for incomplete coagulation. Digest 20 ml. of this filtrate and distil into 10 ml. of *N*/50 acid.

V. *Globulin nitrogen*

Pipette 20 ml. of the casein-free filtrate from (II) into a 50 ml. beaker, add a few drops of bromcresol purple or bromthymol blue solution as indicator and *N*/10 sodium hydroxide solution until the colour denotes a *p*H within the range 6·8–7·2. For normal milk samples 2·0–2·5 ml. are required. Add powdered crystalline magnesium sulphate, 9 g. per 10 ml. of solution, and stir until the solution is saturated. Warming to 25–30° C. markedly assists solution. Set the beaker aside at room temperature for several hours, or, preferably, overnight, and then filter on a small (about 5 cm.) No. 40 Whatman paper that has been very finely pleated to assist filtration. Transfer the precipitate to the paper and wash with saturated magnesium sulphate solution. Transfer the precipitate and paper to a 200 ml. Kjeldahl flask, and digest as in I, except that owing to the presence of some sulphate on the paper, only about 1 g. of potassium sulphate should be added. Distil the ammonia into 10 ml. of *N*/50 acid.

Calculation of nitrogen in the various protein fractions

The following nitrogen percentages have been determined:

I. Total N, i.e. casein + albumin + globulin + proteose-peptone + non-protein N.

II. Non-casein N, i.e. albumin + globulin + proteose-peptone + non-protein N.

III. Non-protein N.

IV. Proteose-peptone + non-protein N.

V. Globulin N.

Thus the total, globulin, and non-protein N are given directly (I, V and III), and the casein, albumin, and proteose-peptone N obtained by difference thus:

Casein N = I minus II.
Albumin + globulin N = II minus IV.
Albumin N = II minus (IV plus V).
Proteose-peptone N = IV minus III.

The procedure is much simplified if it is considered adequate for many purposes to include the proteose-peptone N with the albumin N—as has been done in the past when the presence of proteose-peptone substances was not allowed for—to give a single "albumin" figure. In this case determinations I, II, III and V only are required, providing total, globulin, and non-protein N directly, and by difference, casein N = I minus II, and "albumin" N = II minus III minus V. Total protein nitrogen is given by I minus III, and soluble protein N by II minus III.

The various protein N fractions may be converted into the corresponding amounts of protein by multiplying by 6·38.

The accuracy of the semi-micro method

Determinations of the nitrogen content of the reagents used in this method gave "blanks" varying from 0·05 to 0·10 ml. of *N*/50 acid, with different batches of distilled water and potassium sulphate. A little sulphuric acid was always placed in the water to be boiled as a source of steam for the distillations, to retain any ammonia present. The end-point of the final titrations with methyl red is sharply defined; especially in the cases where the amount of acid necessary for an excess is small, and the concentration of ammonium sulphate in the distillate correspondingly low. The end-point is easily got to within 0·02 ml. of *N*/50 sodium hydroxide.

A series of determinations, on the same milk sample, of the relatively large total nitrogen and non-casein nitrogen contents produced results which agreed to within ±0·2%. The results for the much smaller non-protein, globulin, etc. nitrogen agreed to within ±0·5–1·0%.

A note on the use of selenium as a catalyst in the Kjeldahl digestions

Selenium has been included in the digestion mixture as it was found to reduce markedly the time for digestion, to increase the amounts of nitrogen determined, and to improve the agreement of duplicates. Since Lauro (3) first investigated the use of selenium in Kjeldahl digestions many workers have used it, but some of them (4, 5, 6) have found it to reduce the yield of nitrogen, particularly with cereal products. For this reason the writer made a comparison of the amounts of total nitrogen found in a number of milk samples after digestion of 1 ml. of the milk with 5 ml. of concentrated sulphuric acid, 2 g. of potassium sulphate and 0·2 g. of copper sulphate, with and without the addition of selenium oxychloride; a form of selenium chosen on account of the

ease with which it can be added. The inclusion of selenium resulted in increases of total nitrogen of from 1 to 2% with different milks.

SUMMARY

Details are given of a semi-micro Kjeldahl method for the determination of the total, casein, albumin, globulin, proteose-peptone, and non-protein nitrogen of milk. The method is accurate, rapid, and particularly suitable for the determination of the smaller nitrogen fractions.

The various nitrogen fractions are separated by the improved procedures described in the preceding paper.

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177. THE PROTEIN DISTRIBUTION IN NORMAL AND ABNORMAL MILK

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The analytical methods described and explained in the preceding papers[1] have been applied to a number of normal and abnormal milk samples. The methods permit (*a*) complete separation of casein, (*b*) accurate determination of globulin uncontaminated with casein and albumin, (*c*) determination of the secondary proteins of a proteose-peptone nature as a fraction distinct from the albumin, and (*d*) determination of the true albumin and non-protein nitrogen contents.

The results now obtained provide the first full data on the relative proportions in which all the various protein substances are present in normal and mastitis-infected milk, and in milk with a low content of solids-not-fat. They also show that milk samples of normal appearance, from apparently healthy cows, but persistently low in solids-not-fat content, fall into two classes: (1) pathological cases, with an abnormal protein distribution, from cows with mild subclinical mastitis; and (2) less commonly, physiological cases with a normal protein distribution.

I. THE PROTEIN DISTRIBUTION IN NORMAL MILK

Because of the prevalence of subclinical mastitis, which causes a decrease in the casein and an increase in the soluble protein contents of the milk, the milk samples that have been examined as normal milk were all from cows considered, as the result of bacteriological tests, to be free from the disease. Details are given for ten samples from individual cows, and for six bulk samples.

Description of samples

(*a*) *Individual samples.* A–D were from Shorthorn, E and F from Friesian and G–J from Guernsey cows.

(*b*) *Bulk samples.* K–M were from non-infected Shorthorn cows, and N–P of unknown origin, being samples of "Certified" milk purchased from commercial dairies, and included, not as normal milk, but for comparison with it.

Results

The results for the individual and bulk samples are given in Tables I and II respectively. Each protein is recorded in terms of its N content (protein = N × 6·38), and the distribution of the proteins in each sample is shown by expressing the various protein N figures as percentages of the total N in that sample.

Casein N

In the individual samples casein N averages 78·7 (S.D. 1·0) % of the total N, and varies from 77·3 to 80·7 %. In the bulk samples K–M it averages 78·3 % and varies from 77·7 to 78·9 %. These averages and ranges agree with the figures that the writer has obtained for a large number of individual normal milk samples, the figures for which are not included here as no protein fraction other than casein was determined. It is surprising, therefore, that in the bulk samples N–P casein N accounts for only 76·5–77·4 % of the total N; but, as stated earlier, these samples were of unknown origin and it is probable that they contained some milk from cows with a subclinical mastitis infection. The writer has often found the casein N of ordinary bulk milk samples to account for only 76–77 % of the total N, but has never found the milk from a single uninfected quarter to account for less than 77 %. The data for bulk samples N–P will not be used in calculating the average protein distribution of normal milk.

Albumin and globulin N

Albumin plus globulin N averages 12·4 (S.D. 1·0) % of the total N in the individual samples, and 12·6 in the bulk samples K–M. It varies from 10·3 to 14·1.

Separate albumin N and globulin N figures are available for six of the individual samples, and for the bulk samples. Albumin N varies from 7·5 to 10·7 % of the total N, and averages 9·0 and 9·1 in these individual and bulk samples respectively. Globulin N varies from 2·4 to 4·2 % and averages 3·0 and 3·5 %.

Proteose N

Proteose N varies from 2·8 to 5·3 % of the total N, and averages 4·0 (S.D. 0·7) and 4·1 % in the individual and bulk samples respectively.

Non-protein N

Non-protein N varies from 3·7 to 6·4 % of the total N, and averages 4·9 (S.D. 0·9) and 5·0 % in the individual and bulk samples.

The data for average N distribution are summarized in Table III.

Comparison with previous investigations

The average figure found in this work for the casein N expressed as a percentage of the total N is appreciably greater than the figures reported in the literature. This is to be expected for two reasons, (1) the method used for the precipitation of casein ensured greater casein recoveries than any method used previously, and (2) care was taken not to examine as normal milk any samples which gave evidence of subclinical udder disease. The most detailed investigations of the N distribution in milk hitherto have been made by Davies who used the analytical methods devised by Moir (2), Davies (3, 4) found casein N to account for 76·1 % of the total N in one series, and 76·7 % in another. Golding *et al.* (5) have reported 76·5, Tocher (6) 76·1 and Hoyberg (7) 76·2 %.

Table I. *Nitrogen distribution in samples from individual cows*

	Shorthorn				Friesian		Guernsey			
Cow ...	A	B	C	D	E	F	G	H	I	J
Percentage of milk:										
Total N	0·5437	0·5146	0·5673	0·6236	0·4770	0·5050	0·5346	0·5933	0·6680	0·6220
Casein N	0·4252	0·4045	0·4490	0·4932	0·3705	0·4078	0·4130	0·4617	0·5286	0·4964
Albumin N	0·0511	0·0469	0·0608	0·0590	0·0363	0·0380	0·0698	0·0758	0·0941	0·0748
Globulin N	0·0168	0·0146	0·0134	0·0160	0·0200	0·0140				
Proteose N	0·0212	0·0273	0·0231	0·0287	0·0195	0·0165	0·0229	0·0227	0·0190	0·0221
Non-protein N	0·0294	0·0213	0·0210	0·0267	0·0307	0·0287	0·0289	0·0331	0·0263	0·0287
Percentage of total N:										
Casein N	78·2	78·6	79·1	79·1	77·7	80·7	77·3	77·8	79·1	79·8
Albumin N	9·4	9·1	10·7	9·5	7·6	7·5	13·0	12·8	14·1	12·0
Globulin N	3·1	2·8	2·4	2·5	4·2	2·8				
Proteose N	3·9	5·3	4·1	4·6	4·1	3·3	4·3	3·8	2·8	3·6
Non-protein N	5·4	4·2	3·7	4·3	6·4	5·7	5·4	5·6	4·0	4·6

Table II. *Nitrogen distribution in samples of bulk milk*

Bulk sample ...	K	L	M	N	O	P
Percentage of milk:						
Total N	0·5160	0·5167	0·5190	0·5276	0·5327	0·5344
Casein N	0·4008	0·4050	0·4096	0·4084	0·4105	0·4087
Albumin N	0·0463	0·0445	0·0454	0·0674	0·0699	0·0695
Globulin N	0·0210	0·0212	0·0180			
Proteose N	0·0217	0·0214	0·0206	0·0191	0·0231	0·0278
Non-protein N	0·0262	0·0245	0·0254	0·0327	0·0292	0·0284
Percentage of total N:						
Casein N	77·7	78·4	78·9	77·4	77·1	76·5
Albumin N	9·0	9·6	8·7	12·8	13·1	13·0
Globulin N	4·0	3·1	3·5			
Proteose N	4·2	4·1	4·0	3·6	4·3	5·2
Non-protein N	5·1	4·8	4·9	6·2	5·5	5·3

Table III. *Average nitrogen distribution in normal milk*

Percentage of total N	Individual samples	Bulk samples
Casein N	78·7	78·3
Albumin N	9·3	9·1
Globulin N	3·1	3·5
Proteose N	4·0	4·1
Non-protein N	4·9	5·0

Davies (3, 4) found albumin N to account for averages of 12·6 and 13·4% of the total N. These figures include the proteose N and are to be compared, not with the 9·2% of true albumin in the present data, but with the albumin plus proteose N figure of 13·2%. He also found globulin N to average 6·3 (3) and 4·6 (4)% of the total N, as compared with 3·3% in the present investigation. The differences are due to the use of Moir's method for globulin—which, as already explained (1), gives high results—and to the probable inclusion of a small proportion of mild subclinical mastitis samples.

The improved trichloroacetic acid precipitation for the determination of non-protein N free from any proteose-peptone substances shows that non-

protein N accounts for 5·0% of the total N, as compared with 6·0 and 6·1% reported previously (3, 4).

A new feature of the data given in this paper is the figure for the average proteose N content of normal milk, namely 4·0% of the total N. True albumin plus globulin N account for 75·5% of the N in the total soluble proteins—the so-called "albumin" fraction.

Average N distribution, and protein percentages, in normal milk

From the data presented the average N distribution in normal milk is (when expressed as percentage of total N), casein 78·5, albumin 9·2, globulin 3·3, proteose 4·0, and non-protein N 5·0.

The variation in the total N and protein contents of milk samples from the different breeds of cow makes it difficult to give average figures for the amounts of the several proteins in a typical normal milk. For example, the casein content of the Friesian samples averaged 2·48%, and of the Guernsey samples 3·03%. Taking the average total N percentage in the six different bulk samples as a guide, the figure 0·525 is obtained, a figure in agreement with the generally accepted standard for "bulk" milk. On this basis, average normal milk contains 2·63, 0·31, 0·11 and 0·13% of casein, albumin, globulin, and proteose-peptone substances respectively. The total protein content is 3·18%, and the casein : albumin : proteose : globulin ratios are 23·8 : 2·8 : 1·2 : 1·0.

II. THE PROTEIN DISTRIBUTION IN ABNORMAL MILK

(A) The protein distribution in the milk of cows with mastitis

Three samples of milk from cows with clinical mastitis have been analysed in order to provide data for comparison with the protein distribution in the low solids-not-fat samples to be described in Part II (B).

Description of samples

Cow X was suffering from acute mastitis; the foremilk was clotted and obviously contained a little blood. Cows Y and Z were clinical cases and had only slightly inflamed quarters, but bacteriological examination of their milk showed it to contain large numbers of mastitis streptococci (*Str. agalactiae*) The milk was of normal appearance, and showed no definite alkalinity on bromcresol-purple paper.

Results

The N distribution in the three samples is given in Table IV, along with the fat and solids-not-fat contents in samples Y and Z.

It will be seen that the protein distribution is markedly abnormal. It is well known that in cases of mastitis the composition of the milk tends to approach that of blood, as changes take place in the cells of the mammary gland and permit the passage of unusual amounts of certain blood constituents

Table IV. *Nitrogen distribution in "mastitis" milk*

Cow ...	X	Y	Z
Percentage of milk:			
Solids-not-fat	—	8·12	7·38
Fat	—	3·10	1·90
Total N	0·7248	0·6365	0·4935
Casein N	0·3748	0·4212	0·3300
Albumin N	0·1670	0·0598	0·0654
Globulin N	0·1044	0·0638	0·0423
Proteose N	0·0496	0·0608	0·0319
Non-protein N	0·0290	0·0309	0·0239
Percentage of total N:			
Casein N	51·7	66·2	66·8
Albumin N	23·0	9·4	13·2
Globulin N	14·4	10·0	8·6
Proteose N	6·9	9·5	6·5
Non-protein N	4·0	4·9	4·9

into the milk. The result is the secretion of milk with a low content of the special milk constituents casein and lactose, and a high content of albumin, globulin, proteoses and salts. The greatest increase is in the globulin, which accounts for several times its normal proportion. In the acute case X (Shorthorn) the total N is very high on account of the large soluble protein infiltration. In cases Y (Guernsey) and Z (Shorthorn) the total N is probably slightly reduced.

The deficiency of lactose is reflected in the low percentages of solids-not-fat, 8·12 and 7·38% being very low for the Guernsey and Shorthorn breeds respectively.

(B) The protein distribution in milk with a low content of solids-not-fat

Milk with a low content of solids-not-fat is produced under the following conditions: (1) disease of the udder, e.g. mastitis, as shown in (A); (2) end of lactation, as the solids-not-fat decrease during the usually short drying-off period; (3) prolonged drought, when small decreases in solids-not-fat occur; and (4) in the absence of the above conditions, many cows give milk low in solids-not-fat throughout their lactations, the yields of milk being good. Such cows give normal milk in their first or second lactations, but deterioration of quality is progressive in subsequent lactations (8).

This section of the paper deals with milk samples of the fourth type—samples persistently low in solids-not-fat content. Their occurrence appears, in the light of recent investigations, to be more widespread than is commonly supposed; and, as there is no known method of improving the solids-not-fat content, a serious legal problem is likely to arise. Nicholson & Lesser (9) found that the solids-not-fat content of 64% of 4837 samples of milk from cows under good management failed to reach the presumptive legal standard of 8·5%, the average being 8·3%. Davies (3) determined the protein distribution in a number of these samples, and showed that they were characterized

by low casein and high albumin and globulin contents. The lactose contents were low and the chloride contents correspondingly high. In a second paper (10) Davies discussed his results, and put forward the view that milk of low solids-not-fat content consists of two fractions, (*a*) normal milk, and (*b*) an "isotonic diluent" of albumin, globulin and salts, similar in composition to blood or lymph serum. Davies (10) and Peskett & Folley (11) remarked on the similarity in the composition of the low solids-not-fat milk and mastitis milk. Peskett & Folley (11) stated: "It is our opinion that the poor quality of many of the milks may be due to some change in the cells of the mammary gland, allowing the passage of a transudate resembling oedema fluid into the milk. Such a change appears to be pathological rather than physiological, and might be expected if the mammary gland were damaged, either as a result of previous disease, or owing to an existing condition of mild, chronic mastitis", but there was insufficient evidence to associate the deficiency of solids-not-fat with disease.

Experimental

In attempting to elucidate the problem of persistently low solids-not-fat, it is clearly essential to know whether the problem is fundamentally pathological or physiological. In order to obtain definite evidence, the writer arranged for a series of milk samples of normal appearance, from cows known to have given milk low in solids-not-fat for some time, to be examined for mastitis streptococci on the selective crystal violet-aesculin-blood-agar medium of Edwards (12), and also determined the protein distribution and fat and solids-not-fat contents of the samples.

Description of samples

Samples A–F were from Friesian cows in the herd which provided many of the samples investigated by Nicholson and others. Samples G–I were from Shorthorn cows. None of the udders of these cows revealed clinical signs of disease, and the samples gave a normal reaction on bromcresol purple paper.

Results

The protein distribution, percentage of fat, percentage of solids-not-fat, yield of milk, and time of milking are recorded for each sample in Table V. With the exception of samples C and G, the samples contained mastitis streptococci, some in large numbers.

Discussion of results

Pathologically low solids-not-fat

From the results of the bacteriological examination there is no doubt that cows A, B, D, E, F, H, and I were infected with mastitis of a mild subclinical type. Nevertheless the infection was sufficient to disturb considerably the secretory cells of the gland. The protein distribution figures in Table V show that the milk samples had all the characteristics of mastitis milk. The casein

content was low, and the albumin, globulin and proteose contents were high, particularly the globulin content. In fact these low solids-not-fat samples had the same composition as the samples of milk from cows Y and Z, the mild clinical mastitis cases already described. The solids-not-fat content of the milk from cows A, B, D, E, F, H, and I ranged from 7·78 to 8·62%. Casein N accounted for 65·8–73·5% of the total N, and globulin N for 5·9–8·6%.

Table V. *Nitrogen distribution in milk with a low content of solids-not-fat*

Cow ...	A	B	C	D	E	F	G	H	I
Mastitis infection ...	+	+	−	+	+	+	−	+	+
Percentage of milk:									
Solids-not-fat	7·78	7·89	8·08	8·21	8·47	8·62	7·98	8·28	8·46
Fat	2·00	4·55	2·80	4·00	3·15	3·85	3·25	3·15	3·20
Total	0·4717	0·5237	0·4067	0·5146	0·4884	0·5543	0·4090	0·5223	0·5050
Casein N	0·3194	0·3448	0·3139	0·3550	0·3400	0·3930	0·3174	0·3680	0·3714
Albumin N	0·0544	0·0461	0·0303	0·0499	0·0465	0·0530	0·0322	0·0542	0·0409
Globulin N	0·0277	0·0451	0·0169	0·0383	0·0353	0·0345	0·0176	0·0359	0·0345
Proteose N	0·0397	0·0566	0·0177	0·0451	0·0349	0·0417	0·0203	0·0371	0·0319
Non-protein N	0·0305	0·0311	0·0279	0·0263	0·0317	0·0321	0·0215	0·0271	0·0263
Percentage of total N:									
Casein N	67·7	65·8	77·1	69·0	69·6	70·9	77·5	70·5	73·5
Albumin N	11·5	8·8	7·5	9·7	9·5	9·6	7·9	10·4	8·1
Globulin N	5·9	8·6	4·2	7·5	7·2	6·2	4·3	6·9	6·8
Proteose N	8·4	10·8	4·4	8·7	7·2	7·5	5·0	7·0	6·3
Non-protein N	6·5	6·0	6·8	5·1	6·5	5·8	5·3	5·2	5·2
Milk yield in lb.	6 (p.m.)	9 (p.m.)	28 (p.m.)	14 (p.m.)	10 (p.m.)	6 (p.m.)	29 (a.m.)	9 (a.m.)	8 (a.m.)

Physiologically low solids-not-fat

The bacteriological examination showed that cows C and G had no mastitis infection, and yet the solids-not-fat content of their milk was 8·08 and 7·98% respectively. The total N figures were the lowest of the series, i.e. 0·4067 and 0·4090%, but the protein distributions were normal, as shown in Table V. The protein distribution data show that there had been no dilution of true milk with a transudate containing soluble proteins and devoid of casein, i.e. the samples were not pathological.

A clue to the reason for the low solids-not-fat may be given by the milk yield figures. Cow C gave 28 lb. of milk in the afternoon, and cow G 29 lb. in the morning; both figures correspond with a high daily output. It is reasonable to suggest that the cause of the low solids-not-fat was physiological, these particular cows were unable to produce large quantities of milk without reducing the percentage of solids-not-fat therein. The absolute amount of solids-not-fat was, of course, large; the ability of the animals to manufacture milk proteins and lactose was a limiting factor resulting in the production of a large quantity of milk with a high water content.

However low a milk sample may be in its content of solids, it has the same osmotic pressure as normal milk (13), the osmotic pressure being due mainly to lactose and chlorides which exhibit a complementary relationship (14) and maintain the pressure constant. The physiological low solids-not-fat samples

must therefore have resembled the pathological samples in having low lactose and high chloride contents. The important difference between the two groups was that the healthy cell membranes of the udders of cows C and G were not abnormally permeable to albumin, globulin, and proteose substances, although they permitted the passage of the necessary amounts of salts.

Conclusions

Milk samples of normal appearance, produced by apparently healthy cows and having persistently a low content of solids-not-fat, are of two kinds: (*a*) samples from cows with occult subclinical mastitis, i.e. pathological samples; and (*b*) samples from uninfected cows, the cause of the low solids-not-fat being physiological. These kinds differ in chemical composition. The pathological samples have the low casein and high soluble protein content of mastitis milk, on account of the presence of an isotonic diluent containing only soluble proteins. The physiological cases have a normal protein distribution.

A high proportion of the cases of low solids-not-fat is probably due to mild subclinical mastitis. The published data for the composition of low solids-not-fat milk is undoubtedly data for mastitis milk only (3, 8). A search of the literature has failed to reveal any data for milk with physiologically low solids-not-fat.

The tendency for cows to start the secretion of milk low in solids-not-fat after one or two lactations may well be due to the acquisition of a mastitis infection during those lactations.

III. THE CHEMICAL DETECTION OF SUBCLINICAL MASTITIS

The prevalence of mastitis among the milk-producing herds of this country has certainly been under-estimated. In many cases of chronic infection the disease remains occult for long periods, and cannot be detected by clinical methods such as palpation of the gland and inspection of the milk, or by the simple indirect laboratory tests (cell, catalase, and chloride contents; reaction to indicators) which, when applied to the milk, will detect pronounced infection. Cultural examination of the centrifuged milk sediment on selective media for mastitis streptococci demands much skill, time and labour, and special facilities. Even by this means, a small proportion of mild mastitis cases does not give positive results at every test, and it is necessary to examine several samples of milk drawn at intervals.

Much attention has been devoted to methods for the diagnosis of mastitis, but no simple method that will detect mild subclinical cases has been evolved. The need for such a method is obvious. Subclinical mastitis causes the production of milk of low solids-not-fat content, and an undetected latent infection may become acute, lower the milk yield, and, possibly, result in the loss of one or more quarters. Further, there is evidence (15, 16) that milk from cows with subclinical mastitis produces cheese of inferior quality and value.

On the basis of the data for protein distribution that have been given in Parts I and II, the writer considers that milk from cows with subclinical mastitis can be detected by the casein N/total N ratio. The most convenient form of expression for this is $\frac{\text{percentage of casein N}}{\text{percentage of total N}} \times 100$, giving a figure which, for ease of reference, will be called the "casein number". In the series of normal milk samples described in Part I the casein number was found to average 78·5, and to vary from 77·3 to 80·7. In the course of the determination of the casein number for some other normal milk samples, the minimum figure of 77·0 was found, and this in one sample only. In the series of subclinical mastitis samples described in Part II the casein number ranged from 65·8 to 73·5, indicating how sensitive this number is to the presence of mastitis streptococci.

The definite and significant lowering of the casein number below the minimum for normal milk is well illustrated by the following cases of extremely slight mastitis infection—mastitis in a purely technical sense—which the writer had the opportunity to examine:

(1) Cow R, a Shorthorn, had recovered (as judged by clinical tests) from an attack of mastitis which caused obvious inflammation of the affected quarter. When the milk sample to be described was taken, the total bacterial count was down to a very low figure, and very few mastitis streptococci could be detected. The results of analysis of the mixed milk from the one infected and three normal quarters are given in Table VI.

(2) The total yield of milk from a number of cows that had been shown to harbour a few mastitis streptococci in one or more quarters was collected in one bulk; this milk would have been accepted as perfectly normal. The results of analysis are given in Table VI.

Table VI

	Cow R		Bulk	
	Percentage of milk	Percentage of total N	Percentage of milk	Percentage of total N
Total N	0·4797	—	0·4972	—
Casein N	0·3650	**76·1**	0·3684	**74·1**
Albumin N	0·0483	10·1	0·0522	10·5
Globulin N	0·0160	3·3	0·0234	4·7
Proteose N	0·0275	5·7	0·0244	4·9
Non-protein N	0·0229	4·8	0·0288	5·8

The casein numbers, 76·1 and 74·1, are definitely low in the two samples; and this in spite of the fact that the milk as analysed was a mixture of slightly infected milk with an unknown excess of normal milk.

As a standard of reference, a casein number of 77·0 is suggested for the time being as a useful and lenient minimum for normal milk. The number should reveal both infected milk and the admixture of infected with normal milk. It also serves to distinguish between milk samples with pathological and

physiological low solids-not-fat. If milk from separate quarters of a cow be analysed, the number will be lowered well below 77·0 for any quarter with even an extremely mild infection of subclinical mastitis. In fact the casein number for the milk of any quarter is a valuable index of the milk-secreting efficiency of that quarter. It may be rapidly determined by the method described earlier(1), and for interpretation on the basis given above, the precipitation of the casein should be carried out with the quantities of reagents stated and should be effected as soon as possible, or the milk stored at a low temperature, as it has been found that, in many milk samples—particularly mastitis samples—casein is steadily broken down to proteose and non-protein nitrogenous substances.

After the work that has been described in Parts II and III of this paper had been carried out, it was realized that the encouraging results warranted an investigation on a much larger scale. This is being carried out under the writer's direction, and a large number of milk samples from cows with subclinical mastitis, and from cows yielding milk with a physiologically low content of solids-not-fat, is being analysed. The samples are under strict bacteriological control, and are obtained from the separate quarters of cows of several breeds. The results at the present stage are in full agreement with those recorded above, and when completed will provide an estimate of the relative frequencies of pathological and physiological low solids-not-fat in the different breeds, and provide more data for the selection of a minimum standard casein number.

SUMMARY

The N distribution in terms of casein, albumin, globulin, proteose-peptone, and non-protein N is given for a number of samples of normal milk, of milk from cows with mastitis, and of milk persistently low in solids-not-fat content. Improved methods of determination have been used.

The average N distribution in normal milk is as follows: casein N 78·5, albumin N 9·2, globulin N 3·3, proteose-peptone N 4·0 and non-protein N 5·0% of the total N. The casein N figure is the least variable, ranging from 77·3 to 80·7. On this basis, normal milk contains, as an average: casein 2·63, albumin 0·31, globulin 0·11, and proteose-peptone substances 0·13%. The total protein content is 3·18%, and the casein : albumin : proteose-peptone : globulin ratios are 23·8 : 2·8 : 1·2 : 1·0.

The milk from cows with clinical mastitis is low in solids-not-fat content, and characterized by a decreased content of casein and an increased content of albumin, globulin, and proteose-peptone substances, particularly of globulin.

The milk samples of low solids-not-fat content were of normal appearance and reaction, and produced by apparently healthy cows known to have yielded milk of low solids-not-fat content for some time. The samples were

examined by strict bacteriological methods for the presence of mastitis streptococci, and formed two distinct groups: (1) samples from cows with subclinical mastitis, having the protein distribution of the mastitis samples described above, the cause of the low solids-not-fat being pathological; and (2) samples from uninfected cows, having the protein distribution of normal milk, the cause of the low solids-not-fat being physiological. The two groups of low solids-not-fat cases are discussed, and the conclusion drawn that subclinical mastitis may account for a large proportion of these widespread and puzzling cases.

Many cases of mild subclinical mastitis cannot be detected by the simple chemical tests which will reveal pronounced infection, and satisfactory bacteriological tests, when available, demand much skill and time. For the detection of milk from cows with subclinical mastitis, and for the differentiation of milk samples with pathological and physiological low solids-not-fat, the figure $\frac{\text{percentage of casein N}}{\text{percentage of total N}} \times 100$ is suggested, and the advantage of this "casein number" is illustrated.

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178. A LIPASE (TRIBUTYRINASE) OF COWS' MILK

I. OCCURRENCE, METHOD OF ESTIMATION AND RELATIONSHIP TO LACTATION CYCLE

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(With 6 Figures)

There is no doubt that fat in milk, cream or other dairy products may suffer hydrolysis at ordinary temperatures, with the liberation of free fatty acids. For more than thirty years certain defects in dairy products due to such hydrolysis have been attributed to the presence of a fat-splitting enzyme. How far this enzyme is a constituent of normal, clean milk, and how far micro-organisms are, in practice, responsible for such lipolytic activity are questions not yet solved.

In preliminary experiments carried out some three years ago, we found that triglycerides were, indeed, hydrolysed in presence of milk and milk products, but as the bacteriological conditions of our experiments improved, the amount of hydrolysis diminished. At this stage we began to wonder whether the lipolysis fairly frequently reported in the past was not more likely to be due to fat-splitting micro-organisms, alive or dead, or enzymes liberated from them, rather than to an enzyme originally present in the milk as secreted.

On looking carefully into the conditions under which previous experimental work on the inherent lipolytic enzymes of milk had been carried out, it became clear that whilst some investigators had adopted reasonable precautions to avoid bacterial contamination, several had used methods which were seriously open to question. (For a summary of earlier work see Nair(1) and Rice & Markley(2).) Amongst the procedures which seemed to us dubious are the following:

(1) The incubation of admittedly non-sterile material suspected of containing a lipase with fat for long periods of time, frequently under conditions almost optimal for bacterial reproduction.

(2) The estimation of lipolysis by titration of increase in the *total* acidity of a reaction mixture, where processes other than lipolysis, as possible sources of the increase in acidity, had not been excluded.

(3) Reliance on antiseptics not previously proved to be efficient.

(4) Use of antiseptics which seriously inhibit lipase action.

(5) Use of substrates other than triglycerides for the estimation of lipolytic activity.

After examining the admissible evidence we came to the conclusion that it was very desirable that, as our first objective, we should verify earlier claims regarding the presence in fresh, clean cows' milk of a preformed, triglyceride-splitting enzyme.

EXPERIMENTAL

It was evident that we should have to look for an enzyme present, at best, in relatively small amount. To establish its presence or absence with certainty, and particularly if reasonably quantitative measurements were to be made, methods would have to be used which were delicate and precise.

(1) *Sucrose-cream substrates*

The obvious substrate for the possibly lipolytic activity of milk is milk fat, and our first experiments were made using the sucrose-cream substrate of Nair(1) following the method of Rice & Markley(2). Our results were similar to those found by these investigators, but the length of time required for measurable reaction to take place in this medium, the time occupied in weighing aliquots, and (in our experience) the unsatisfactory microbiological condition of the mixtures at the end of the protracted reaction time made this method unsuitable for our purpose.

(2) *Glycerol-cream substrates*

Glycerol-cream substrates or glycerol-buffer-milk substrates were then tried, together with modifications in the method of fatty acid titration.

Two experiments only will be described:

(*a*) *Heated glycerol-cream* (1 vol. cream to 1·5 vol. glycerol) was incubated with differing quantities of milk at 37° C. for 18 days. The titration results (baryta, on approximately 20 g. aliquots) and the bacteriological control (plate counts on Standard Milk Agar) figures are shown in Table I.

Table I. *Action of milk enzyme on glycerol-cream substrate*

No. of solution	% milk	Titrations calculated as ml. 0·1 *N* $Ba(OH)_2$ per 100 g. mixture			Bacterial counts: colonies per ml.			
					Original		After 17 days	
		Original	17 days later	Difference	Incubation 37° C. for 4 days	Incubation 22° C. for 5 days	Incubation 37° C. for 4 days	Incubation 22° C. for 5 days
1	None	3·13	4·79	1·66	170	160	90	180
2	1	3·36	4·85	1·49	200	170	40	50
3	5	3·68	5·81	2·13	350	500	70	40
4	10	5·06	6·99	1·93	370	970	50	170
5	20	6·27	8·85	2·58	390	1380	70	140
6	20 (boiled)	6·34	6·89	0·55	240	410	20	70

(*b*) *Heated buffer-glycerol* was incubated with clean raw and boiled milk as follows.

Two mixtures each containing 60 ml. 0·1 *M* barbiturate buffer at *p*H 8·5,

100 g. glycerol and 50 ml. milk, in one case raw and in the other boiled, were made up in sterile flasks, placed in a thermostat at 37–38° C., shaken gently and continuously for about 24 hr., then transferred to a 37° C. incubator for the remainder of the experimental period. The two flasks were given an occasional shake. Steam distillations were made on aliquots at 0 and 82 hr. and after 10 days. The results are shown in Table II.

Table II.

	Titres of distillate ml. *N*/100 NaOH		*p*H of solutions		Bacterial count: Raw				Bacterial count: Boiled			
Time	Raw	Boiled	Raw	Boiled	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*
0 hr.	1·27	1·15	8·50	8·50	800	480	480	0	140	10	0	0
82 hr.	6·65	2·45	—	—	—	—	—	—	—	—	—	—
10 days	13·90	3·70	7·60	7·94	0	0	—	—	0	0	—	—

a = No. of colonies per ml. found by plating on standard milk agar.
b = No. of colonies per ml. found by plating on tributyrin agar.
c = No. of lipolytic colonies shown on tributyrin agar.
d = No. of lipolytic colonies showing area of digestion with diameter of 2 mm. or above.

When the distillation from 20 ml. of solution was made at the end of 10 days the distillate had a quite characteristic volatile fatty acid smell. Droop Richmond (3) determinations on the distillate gave evidence of the presence of solid volatile fatty acids, which were also observed in small quantities in the condenser. The titration curve was otherwise very similar to that given by Droop Richmond for butyric acid. The very slight increase in titre shown in the boiled milk aliquots may be due either to the formation of volatile acids from traces of impurities in the glycerol or to the formation of lactic acid from the lactose of milk. Lactic acid is slightly volatile in steam and its presence in the distillate from both these experimental solutions was proved.

The results of these and of other similar experiments could only be interpreted on the assumption that fresh milk contained an enzyme capable of splitting fats. Clearly, however, it was present in amounts which this method was insufficiently sensitive to estimate in any quantitative way.

Method for determination of tributyrinase

As a result of numerous trials which need not be detailed here, a method has been devised which enables us to obtain, in from 4 to 6 hr., a degree of hydrolysis large enough to be measured with fair accuracy, and thus provides a standard method of determining differences in enzyme content between different milks or milk fractions. It consists in the titration of butyric acid formed under standard conditions of time, temperature, acidity and volume from the action of the enzyme in milk on tributyrin in a buffer solution. To increase sensitiveness and avoid certain difficulties, the butyric acid formed is distilled off before titration. Suitable controls are carried out at the same time.

The *buffer* used is sodium diethyl barbiturate (4). The *p*H of optimum activity of the milk tributyrinase under the conditions of our standardized

method has been found to be between 8·2 and 8·7 (see Fig. 1). A *p*H of 8·5 is therefore used.

The *substrate*, tributyrin, is soluble to the extent of 1 part in 8700 in water at 30° C.(5). Its solubility in the buffer solution at 37° C. we have found to be 1 part in 2800 of solution. Taking Sobotka & Glick's(5) findings for the increased solubility of tributyrin in lipolytic enzyme solutions (from the liver and pancreas) it is likely that our substrate has an even greater solubility in the reaction mixture containing milk, than in the buffer solution alone. The use of tributyrin appears, therefore, to avoid some of the major difficulties met with when enzyme and substrate are not soluble in the same phase. Large excess of undissolved substrate is to be avoided.

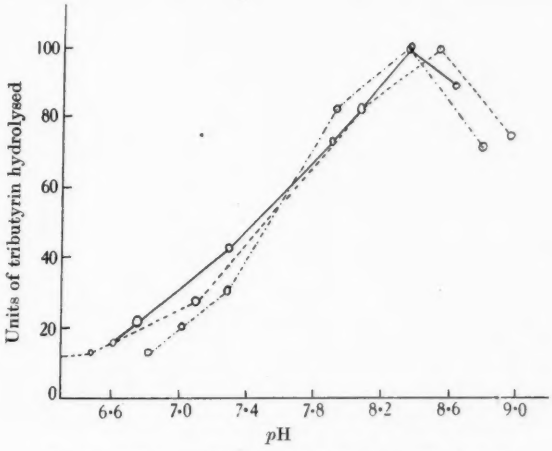

Fig. 1. Optimum *p*H for milk tributyrinase (Na diethyl-barbiturate buffer).

A typical reaction mixture consists of:

100 ml. *M*/10 sodium diethyl barbiturate.

0·5 ml. tributyrin (pure).

20 ml. milk or other enzyme—containing material placed in 150 ml. Erlenmeyer flask.

No antiseptic is used. A number of preliminary experiments using toluene, thymol, hydroxyquinoline sulphate, etc. convinced us of the inadvisability of using such methods of preventing bacterial growth. This is controlled, instead, by plate counts, before and after the experimental period, on standard milk agar. In the more recent experiments these have also been carried out on tributyrin agar. During the experimental period of not more than 6 hr., bacterial proliferation, and particularly the proliferation of tributyrinolytic organisms, is found to be small.

From the reaction mixture a 20 ml. aliquot is removed at once and transferred to a 250 ml. round-bottomed pyrex glass flask for steam distillation,

the reaction being stopped and the acidity increased to a *p*H of approximately 1·6 by the addition of exactly 10 drops of phosphoric acid (A.R., sp. gr. 1·75).

The rest of the mixture is kept in a water thermostat at 37° C. for the experimental period, with gentle and continuous mechanical shaking. Aliquots of 20 ml. are withdrawn at intervals, acidified and distilled.

The distillation apparatus, made of pyrex glass with ground glass joints (Fig. 2) gives reliable duplicate results, which are not obtained when cork or rubber stoppers are used for the connexions. Before a distillation is carried out, the apparatus is steamed through until a volume of 100 ml. of distillate

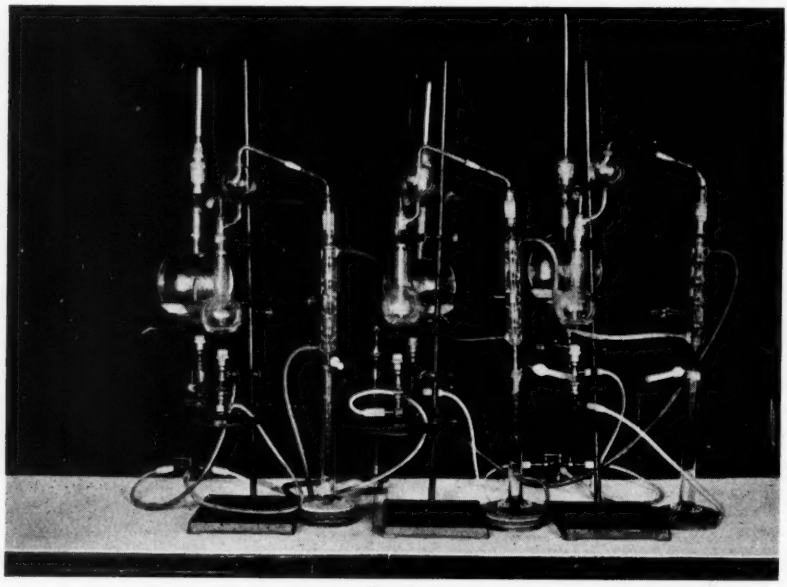

Fig. 2.

collected in the cylinder (fitted to the condenser with a two-holed rubber stopper, carrying a soda-lime tube) gives, on titration with CO_2-free $N/100$ NaOH, a value less than 1 ml.

The water distillation flask is removed and replaced by one containing the acidified reaction mixture, and 100 ml. of distillate collected while the volume in the reaction flask is kept as nearly as possible constant at 20 ml. by adjusting a small burner beneath the flask. The distillate is titrated from a micro burette with $N/100$ NaOH, using phenolphthalein as indicator, in the cylinder in which it has been collected, a glass plunger being used for mixing.

According to Willstätter & Memmen (6) tributyrin may be contaminated with dibutyrin. Reaction mixtures tested under the same conditions with tributyrin as ordinarily used and after purification according to the method

recommended by these authors gave hydrolysis curves which were identical within the error of the experiment.

It was thought just possible, after consideration of the facts published by Hiscox & Harrison(7), that small quantities of butyric acid, liberated by the tributyrinase, might be retained by the fat or other constituents of the substrate. These workers found that on steam distillation there was marked retention of some of the volatile acids by unhydrolysed butterfat, but in the case of butyric acid more than 90% could be recovered by collecting three times the original volume. To ensure as complete recovery as possible we collect five times the volume. By the addition of a known weight of sodium butyrate to our reaction aliquot, we find that over 91% of the added butyric acid is collected in the first 100 ml. of the liquid steam-distilled under our standard conditions from 20 ml. of the mixture. There is thus no serious retention of the acid by the reaction mixture.

In another series of experiments, distillates from reaction mixtures in which enzymic activity had proceeded for several hours were fractionally re-distilled in a Duclaux apparatus. The titres of each fraction were plotted and gave curves identical with those obtained for pure butyric acid under the same conditions.

Effect of shaking.

Under our experimental conditions, gentle shaking increases somewhat the rate of hydrolysis of tributyrin by milk. Typical results are given in Table III.

Table III. *Effect of shaking on rate of hydrolysis of tributyrin by cows' milk. Corrected titres of distillates from* 20 *ml. aliquots*

Time min.	Shaken continuously	Shaken for 90 min., then not shaken, duplicate flasks		Not shaken for 90 min., then shaken, duplicate flasks	
0	0·0	0·0	0·0	0·0	0·0
	Shaken	Shaken		Not shaken	
90	8·7	8·9	8·7	5·3	5·4
	Shaken	Not shaken		Shaken	
300	20·0	17·0	16·4	20·9	21·3

Thus shaking accelerates hydrolysis to an appreciable, but not to an overwhelming, extent.

Rate of shaking.

Usually the speed of shaking was 48–52 (double) movements per min. An experiment in which the speed of shaking was varied from 36 to 70 movements per min. gave no appreciable effect on the rate of hydrolysis. It is possible that shaking speeds of considerably less than 36 movements per min. would be sufficient to maintain an optimal rate of hydrolysis, and that all that is necessary is sufficient movement of the reaction fluid to keep the liquid

saturated with tributyrin, slight excess of which normally remains at the bottom of the reaction flask.

Changes in volume of fluid in the distillation flask during the steam distillation

A series of experiments showed that the volume of liquid in the distillation flask (20 ml. at the beginning) could be varied somewhat without affecting the titre of the distillate. It is advisable to keep this volume at about 20 ml. but it can be increased to 30 ml. or even slightly more without trouble. It should not be allowed to fall below 15 ml.

By adjustment of a small gas flame below the distillation flask it is an easy matter to maintain the volume well within these limits.

Controls and blanks

(1) *Blanks.*

In early experiments distillations of two control solutions, one without tributyrin and one without milk, were made simultaneously with those containing the milk under investigation; and the titration values for these solutions were subtracted from those of the experimental solution; in some cases boiled or sterilized milk was used to control the raw milk. None of these solutions represents the actual conditions in the experimental flasks, and their inclusion necessitates the use of considerable quantities of reagents and a prolonged period of time for distillation. It was decided that the value of an aliquot taken immediately the solutions are made up (i.e. an estimate of any acids originally present which are volatile in steam, which can be subtracted from the final value) is the more reasonable control, as the time and conditions of the experiment do not encourage bacterial growth, nor the formation of volatile acids other than by the enzymic breakdown of the tributyrin. Any acid titre resulting from the steam distillation alone is controlled by collection and titration of 100 ml. of water before every experimental distillation. The great majority of the "water blanks" are below 0·40 ml. of $N/100$ NaOH.

(2) *Control of solutions during incubation period.*

(*a*) *Reaction.* The reaction and bacterial content of experimental solutions must be kept as unchanged as possible throughout the incubation period of 6 hr. *p*H determinations (generally using a glass electrode) have been made on samples of the original milk and the solution in experimental flasks taken at the beginning and end of the incubation period and plate counts have been made for bacterial content, on standard agar, and, in order to determine the number of lipolytic colonies, on tributyrin agar also.

The variations of reaction during the experimental incubation period are indicated by the figures in Table IV.

Table IV. *pH of original solutions and changes during experimental period of 6 hr. Figures show numbers in each category*

*p*H below	*p*H	*p*H above
	Original milk	
6·42	6·42–6·78	6·78
37	168	48
	Reaction mixture at start	
8·4	8·4–8·6	8·6–8·7
14	241	3
	Change in reaction during incubation	
0·3 *p*H or less	0·3–0·5 *p*H	Above 0·5 *p*H
229	24	5*

* Three of these were colostrum experiments

(*b*) *Bacterial content.* Increases in bacterial content during incubation were small, and in view of the shortness of the experimental period can be looked upon as negligible (see Table V).

Table V. *Bacterial content of original reaction mixtures, and the same after the 6 hr. experimental period*

	No. of samples	Below 5000 per ml. %	Below 20,000 per ml. %	Below 100,000 per ml. %	Above 200,000 per ml.
Original milk	296	88·9	97·3	99·3	1 sample
Solutions before incubation	306	94·4	98·7	99·7	1 sample*
Solutions after 6 hr. incubation	306	88·6	96·1	98·7	1 sample*

* In this sample the colonies were uncountable before incubation, and increase could not be estimated.

It may be concluded that neither the changes in *p*H or in bacterial population during incubation were such as to interfere with the validity of the experimental findings.

Tributyrinase in milk of individual cows

Samples from the morning milking of 45 cows in the Institute herd were tested. The animals included were Guernseys or Shorthorns in their first to tenth lactations. Samples were taken between the first and 505th day of lactation. All samples were found to contain the .zyme, the titration values of 100 ml. of distillate with $N/100$ NaOH after 6 hr. incubation (omitting colostrum values) varying from 3·8 for Lottie 8, a Shorthorn cow in her first lactation at 505 days, to 23·5 for Dora 4, also a Shorthorn cow in her fourth lactation at 161 days. Still greater activities have been met with in colostrum and in the milk of very early lactation.

Change in enzyme content during lactation period.

Estimations of the tributyrinase content throughout the course of lactation were made in several cases, and showed that in colostrum the content is very high compared with its value in mid and late lactation. During the first few days the amount decreases rapidly; it then rises to a value which does not vary greatly thereafter throughout lactation, until, after 300 days (or in many cases immediately before drying off) the values show a slight decrease. This result was unexpected, as in several publications in which reference is made to a fat-splitting enzyme in milk the statement is made that it occurs in increased amounts in the milk of late lactation.

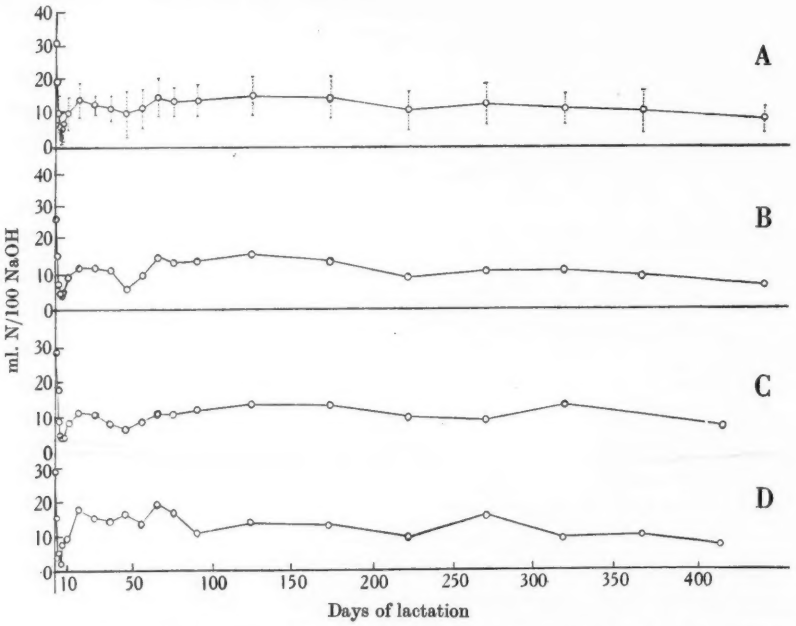

Fig. 3. Tributyrinase values of milk samples plotted against number of days of lactation. *A*, "Average" curve, all cows. Standard deviations shown. *B*, Median curve, all cows. *C*, "Average" curve, Shorthorn cows. *D*, "Average" curve, Guernsey cows.

Fig .3 *A* shows 320 milk tributyrinase values plotted against the number of days of lactation. The curve, as might be expected, is similar to that given by a number of individual animals which have been followed through a lactation. The enzyme was present in all samples of milk, but the highest values were invariably obtained in the first few days of lactation. In no case was there an appreciable increase towards the end of the period among the normal samples with which we are dealing. The standard deviation at each point is shown. A median curve for all cows, and average curves for Shorthorn and Guernsey groups separately, show no essential variations (Fig. 3 *B*, *C*, *D*).

Seasonal variation.

Our figures so far show no marked seasonal variation in the amount of tributyrinase present in cows' milk. It should perhaps be pointed out that the animals investigated are all healthy and in first-class nutritional condition throughout the year.

Attempts at correlation of tributyrinase content with the amounts of other milk constituents.

(*a*) *Fat.* The possibility of correlation of the triglyceride-splitting enzyme with the fat content of the milk (Gerber) was considered. Mean figures for the percentage fat content of 327 samples are shown, plotted against the enzyme content, in Fig. 4.

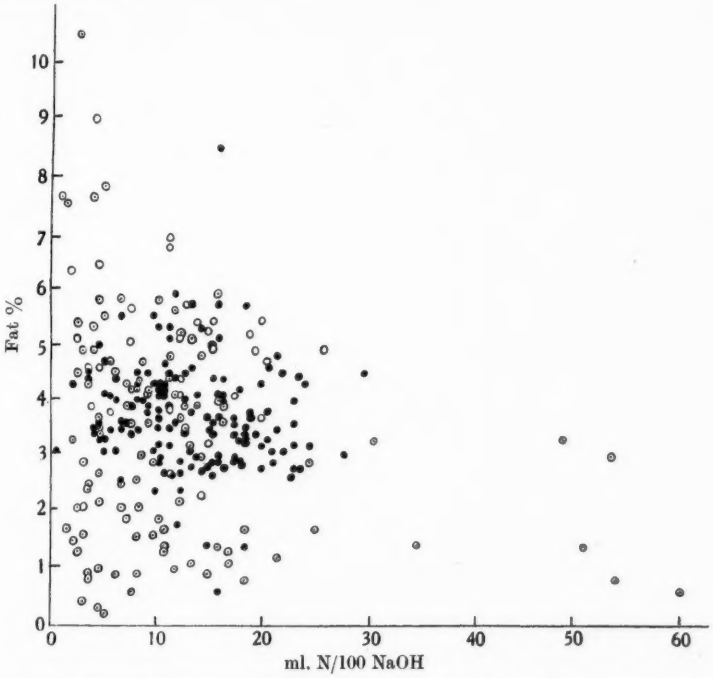

Fig. 4. Tributyrinase values of 327 samples of milk plotted against percentage of fat.

⊙ Under 10 days' lactation. ● 10–249 days' lactation.
○ 250 days' lactation and above.

The fact that the enzyme is not apparently associated with the fat of the milk has been confirmed on several occasions by estimations of its activity in whole milk, and in the separated cream and skim milk from the same sample. Using the same quantities in each flask the value for separated milk is always found to be higher than that for whole milk and considerably higher than that for cream: this is true whether the cream is allowed to rise naturally (in a

large separating funnel), is separated in the dairy, or is centrifuged off in the laboratory (Table VI).

Table VI. *Distribution of enzyme between the fat and the aqueous phase of milk (ml. N/100 butyric acid produced in 20 ml. of reaction mixture)*

Treatment	Whole milk	Separated milk	Cream
Allowed to rise naturally	7·5	11·70	2·1
Centrifuged in the laboratory	10·0	11·4	5·0
Centrifuged in the laboratory	27·2	29·4	15·8
Separated at dairy	12·6	14·3	6·1

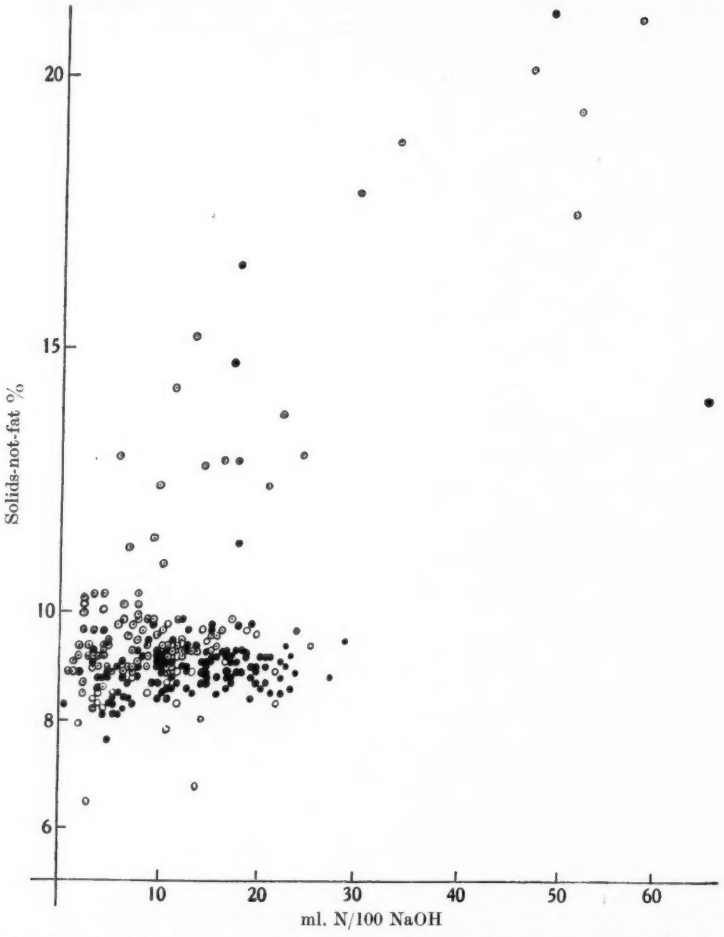

Fig. 5. Tributyrinase values of 247 milk samples plotted against percentage of solids-not-fat.
⊙ Under 10 days' lactation (including some samples of colostrum).
● 10–249 days' lactation. ○ 250 days' lactation and above.

(*b*) *Solids-not-fat.* Fig. 5 in which the figures for 247 determinations of solids-not-fat (determined by evaporation and weighing) are plotted against the

enzyme content figures of the same sample does not reveal any correlation between these properties except with samples taken very early in the lactation period (including samples of colostrum).

(*c*) *Phosphatase content.* Estimations of phosphatase, made on 267 samples, show no correlation with tributyrinase (Fig. 6).

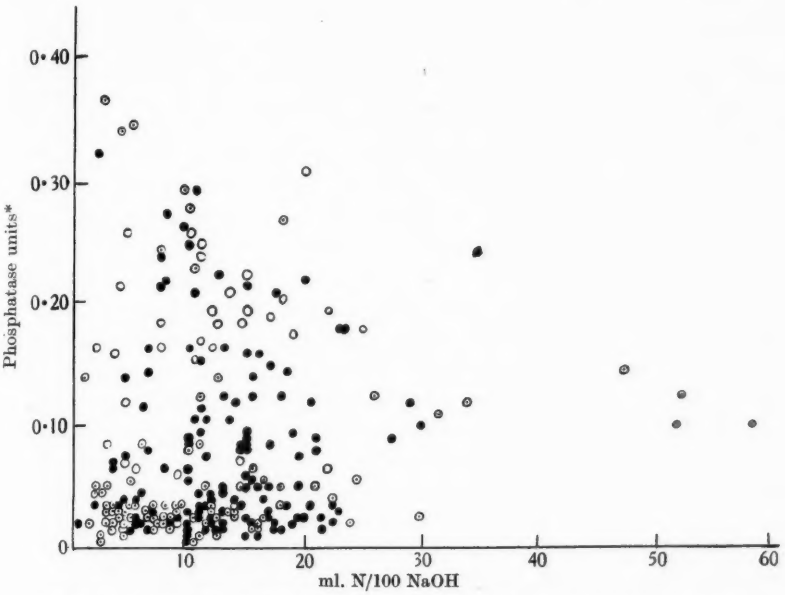

Fig. 6. Tributyrinase values of 247 milk samples plotted against phosphatase values.

⊙ Under 10 days' lactation. ● 10–249 days' lactation.
○ 250 days' lactation and above.

* Kay, H. D. & Graham, W. R. (1933): *J. Dairy Res.* **5**, 65.

Thermostability of tributyrinase

Some preliminary experiments have shown that the tributyrinase of milk obtained in the middle of an ordinary lactation period is destroyed fairly easily by heat. Table VII gives typical findings.

Table VII. *Milk held for* 30 *min. and then cooled*

Sample 1		Sample 2	
Temp. of heating ° C.	Destruction of enzyme %	Temp. of heating ° C.	Destruction of enzyme %
45·5	41	45·5	43
49	65	49·5	73
53	92	53·5	93
56	93	56	98+

The enzyme would appear to belong to the more thermolabile of those found in milk. It is, for example, distinctly more easily destroyed than phosphatase (Kay & Graham (8)), or than peroxidase (Spitzer & Taylor (9)), but has a thermostability of a similar order to that of amylase (Orla-Jensen (10)).

Discussion

After re-reading the literature in the light of our own experiments on sucrose-cream substrates and sucrose-glycerol substrates, we are satisfied that a "true lipase", i.e. an enzyme that will hydrolyse naturally-occurring fats, is present in fresh milk. Apart from our own experiments, those of Rice & Markley (2) and of Nair (1) and particularly those of Dorner & Widmer (11) are very difficult to explain on any other basis. The activity of the "true" lipolytic process in cows' milk is, however, very small when compared with that of many tissues and tissue extracts.

Whether the enzyme which we have shown to be present in fresh milk and which hydrolyses tributyrin fairly readily is *ipso facto* a lipase or not is a question of definition. Since tributyrin is probably not a naturally occurring fat, an enzyme which hydrolyses this substrate and this substrate only would, by the strictest definition of the term "lipase", be excluded. What is really of importance is whether the enzyme we call "tributyrinase" is, in fact, identical with the "true lipase" of milk. If so the former, which may be fairly easily determined quantitatively, may be taken as a measure of the latter. Definite evidence on this point is not yet available.

The apparent low activity of the "true lipase" in milk is, as Dorner & Widmer's findings indicate, due largely to the state of division of the substrate. If the size of the milk-fat particles is further diminished and their area increased, the rate of lipolysis also increases very markedly. In tributyrinase systems, the substrate is in solution—a state in which the size of fat particle may be regarded as minimal—and the activity of the enzyme, if it is indeed identical with milk lipase, would be expected to be (in so far as this factor is concerned) optimal.

From the experiments described in this paper a little has been learned about milk tributyrinase. In any comparison between this enzyme and milk lipase it is probably our knowledge of the latter that is the limiting factor. We are as yet very far from being able to say that milk tributyrinase and milk lipase are identical, but it is noteworthy that, as far as our present knowledge goes, the distribution of the two enzymes between the fat and the aqueous phases of milk is similar and that the thermostability of the two enzymes is also similar (compare, for example, our results with tributyrinase with those of Sharp & de Tomasi (12) for lipase). The substrates for their activity are, of course, closely related chemically, and the *p*H zone of maximal activity of the two—again within the bounds of available data—is in a similar region. Apart from the doubtful increase of "true lipase" towards the end of the lactation period,

no points of dissimilarity between the two enzymes have yet been found; further work may, of course, disclose them and show clearly that the two enzymes are distinct.

That milk tributyrinase is related to milk phosphatase (also an esterase) seems, on the other hand, to be already disproven. The most striking point of difference between the two is, perhaps, that whilst tributyrinase is present to a much larger extent in the aqueous than in the fatty phase in milk, the opposite is true for phosphatase. Moreover, milk phosphatase rises rapidly in concentration in end-of-lactation milk, whilst tributyrinase tends to fall at this period.

SUMMARY

An enzyme which hydrolyses tributyrin is present in all samples of fresh cows' milk examined. A method has been developed for the determination of the tributyrinase activity of a given milk or fluid.

In milk the enzyme is present in the aqueous rather than the fatty portion. Its optimal range of activity is in the region *p*H 8·2–8·7. It is rather more thermolabile than phosphatase.

Its concentration in milk varies considerably from cow to cow, and in individual healthy cows there is a fairly regular variation throughout the lactation cycle. It is highest in concentration in colostrum, then the concentration falls to a minimum at about 10 days, rising later to a figure intermediate between this minimum and the colostral value. It shows no sign of increase towards the end of the lactation period.

Its relationship to the "true lipase" of milk is discussed.

We are much indebted to Dr A. T. R. Mattick for help in the microbiological control of our experiments. We also wish to record the valuable laboratory assistance we have received from Mr H. Hallett, Mr A. Wagstaff and Mr N. Gruber.

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179. ANNATTO AS A CHEESE COLOUR

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(With 2 Figures)

The properties and the methods of standardization of annatto cheese colour have been under investigation in this laboratory for some time, and work on the subject is still in progress. It was intended to withhold publication until completion of the study, more particularly until the colour of a large number of different samples of annatto, measured under various conditions, had been compared with the colour of cheeses made with these samples, and until the influence of conditions within the cheese, e.g. fat-content, moisture-content and *p*H on the depth of colour produced by annatto, had been investigated. In the Appendix to a recent paper, however (Barnicoat (1)), certain statements have been made with reference to annatto and its standardization which are so much at variance with the results already obtained here that it has been thought advisable to put forward the present preliminary paper.[1] The criticisms of Barnicoat's conclusions are based on results obtained with a Zeiss-Pulfrich Gradation Photometer.

On the basis of these observations, some suggestions are made on the subject of the standardization of commercial annatto and of the colour of cheese itself. In New Zealand, cheese factories receive a premium for coloured cheese, and the amount of the premium is varied according to the depth of colour of the cheese. At present, there are three classes of coloured cheese, but the classification is based only on a judgment of the colour by a grader. The advantage of having some form of colour standard for cheese is thus obvious. It is submitted that the form of colour standard described below would be suitable for commercial use.

Effect of dilution on the colour of annatto solutions

The transmission curves of ten samples of annatto, made by five different manufacturers, were examined in the Gradation Photometer, over a range of dilutions from 1/10,000 to 4/10,000 in $N/10$ NaOH, using the Zeiss S-Filters. For each sample, the shape of the "Typical Colour Curve" (logarithm extinction coefficient) remained constant throughout this range. A typical transmission curve of annatto at a dilution of 2/10,000 in $N/10$ NaOH is given in

[1] Publication has been made all the more necessary since Davies (2) apparently has accepted the conclusions of Barnicoat without question.

Fig. 1 (whole line). A 1 cm. cell was used in all cases. The extinction coefficients (-logarithm transmission) with the S 47 Filter (maximum absorption) were plotted against concentration for each sample, and in every case the results followed Beer's law within experimental error ($\pm 1\%$). These results thus do not show agreement with the statement of Barnicoat that "the intensity of colour (in transmitted light) is not quite proportional to the dilution, i.e. annatto solutions do not follow Beer's law strictly". Barnicoat based his conclusion on measurements with the Lovibond tintometer, and found that the graph of total Lovibond units (yellow + red) plotted against concentration did not give the straight line which Beer's law demands. In reaching his conclusions, Barnicoat appears to have overlooked certain characteristics of

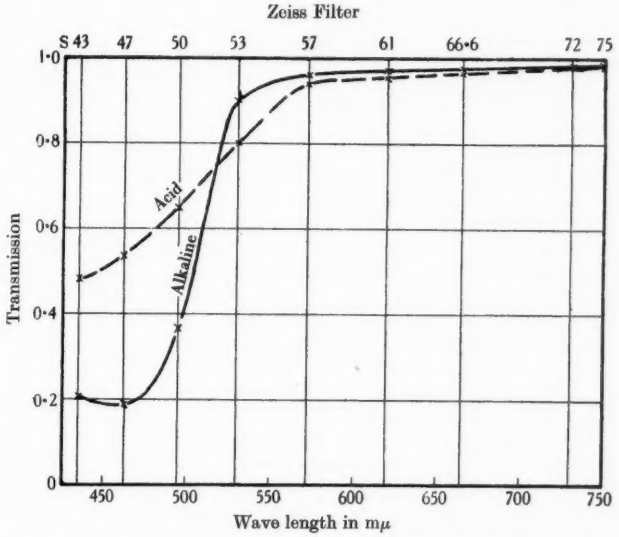

Fig. 1. Transmission curves of annatto solutions, 2/10,000 dilution, 1 cm. cell. Whole line, $N/10$ NaOH. Broken line, 0·6 molar acetate buffer, pH 5·5.

the Lovibond glasses. Gibson & Harris (3) in their study of the Lovibond glasses have shown that for any given wave-length of light,

$$\text{Extinction coefficient} = -\log T_\lambda = kN + b,$$

where T_λ = transmission at the given wave-length, N = the Lovibond number for a given series, k, b = constants.

The value of k depends on the spectral densities of the unit Lovibond glasses. The value of b depends mainly on the loss of light by reflexion at the surfaces of the glasses. Gibson & Harris found that, owing to this reflexion, the hypothetical No. 0 Lovibond glasses transmitted only about 90% of the incident light. This means that 100% transmission, as would occur with zero concentration of a dye, would be represented by a negative Lovibond reading.

The Neutral Lovibond glasses, of course, give some measure of compensation for this effect. Gibson & Harris found, however, that although the value of k was practically constant for the red and blue series, for the yellow series it was higher in the lower members of the series than in the higher members. Without details of Barnicoat's readings (including his Neutral readings), it is impossible to give an exact analysis of his results, but both the characteristics of the Lovibond glasses referred to above would tend to convert the straight line which Beer's law demands into a curve such as Barnicoat obtained.

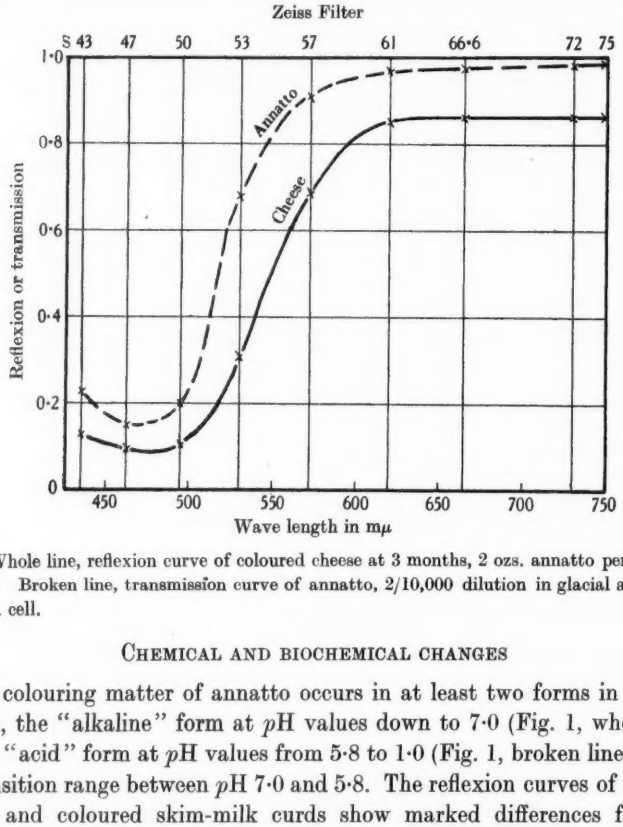

Fig. 2. Whole line, reflexion curve of coloured cheese at 3 months, 2 ozs. annatto per 1,000 lb. milk. Broken line, transmission curve of annatto, 2/10,000 dilution in glacial acetic acid, 1 cm. cell.

Chemical and biochemical changes

The colouring matter of annatto occurs in at least two forms in aqueous solution, the "alkaline" form at *p*H values down to 7·0 (Fig. 1, whole line), and the "acid" form at *p*H values from 5·8 to 1·0 (Fig. 1, broken line). There is a transition range between *p*H 7·0 and 5·8. The reflexion curves of coloured cheeses and coloured skim-milk curds show marked differences from the curves of both these forms (the S 50 value (on the Gradation Photometer) of the "alkaline" form is always higher than the S 43 value, while the S 50 value of coloured cheese is always lower than the S 43 value), but resemble very closely the curves of annatto dissolved in non-ionizing solvents such as glacial acetic acid, toluene or xylene (Fig. 2). The fact that both skim-milk curd and pulped filter paper dyed with annatto give the same type of curve, shows that it is not the carotene but the annatto which is responsible for the shape of the reflexion curve of whole-milk cheese. It is thus obvious that the

annatto is present in cheese in a form which does not occur in aqueous solution. In the author's view, therefore, it is not justifiable to assume that results of experiments carried out on one form of annatto in solution will apply to a completely different form of annatto combined with the solid casein of cheese. The validity of Barnicoat's conclusions in this section of his paper, based, as they are, on experiments with buffered solutions of annatto, are therefore open to serious question.

ANNATTO IN THE CHEESE-MAKING PROCESS

The conclusion of Barnicoat that annatto in cheese is mainly in combination with the casein has been confirmed in this laboratory. Fat was extracted from the cheeses by grinding with toluene in the cold. It had previously been found that toluene did not extract the annatto from its combination with casein. Colorimetric comparisons of the extracted fats of coloured and uncoloured cheeses showed that only a negligible quantity of the annatto was dissolved in the fat of the coloured cheeses. This provides an explanation of the occasional appearance of mottled colour in coloured cheeses made from high-testing raw milk. The more lightly coloured free fat can be seen at the edges of the curd particles.

The results cited by Barnicoat on the use of different concentrations of the same annatto in the cheese-making process (Table III of his paper), in the present author's view, do not support the conclusion that there was "a proportionality between the colour of the cheese and the initial concentration of annatto in the milk".

With approximately 0·6 oz. of annatto per 1000 lb. of milk, Barnicoat found that the cheese at one month showed an advance in colour of 2·1 Lovibond units (yellow + red) over the uncoloured control; with twice this amount of annatto, the advance was 6·9 units; and with four times this amount, the advance was 9·1 units. The first 0·6 oz. portion of annatto added 2·1 Lovibond units; the second added a further 4·8 Lovibond units; and the last 1·2 oz. portion added 2·2 Lovibond units, or 1·1 units per 0·6 oz. portion. It is considered that these results do not indicate reasonably accurate proportionality, even if the characteristics of the Lovibond glasses are taken into consideration.

In referring to the "red and yellow components of the annatto pigment", Barnicoat seems to imply that annatto consists of two components whose colours correspond to the red and yellow of the Lovibond scale respectively. He appears to confuse the constituents of the annatto itself with the constituents of the light reflected from the casein dyed with annatto. He states, for example ((1), p. 70), that "'pink' discoloration is produced merely by the removal of some of the yellow component of the pigment", whereas actually his results show only that the dyed casein has undergone an alteration such that it reflects more of the blue-violet (as measured by the Lovibond yellow)

while its reflexion of green (as measured by the Lovibond red) remains constant. His statement, as it stands, is liable to misinterpretation.

Storage of annatto solutions

The recommendation of Barnicoat that annatto be stored in light-proof glass containers is supported only by his experiments showing that iron and copper accelerate the oxidation of annatto by hydrogen peroxide. He has not published any experiments which show that, under normal conditions of storage, annatto is more stable in glass than in the steel drums used in ordinary commercial practice. Actually, owing to surface action, annatto stored in glass bottles deteriorates more quickly than in steel drums, a film of deposit being noticeable on the inside surface of the glass. Samples kept in this laboratory in glass bottles in the dark have lost as much as 5% of their strength in a month as compared with no loss in the bulk sample of annatto stored in a large, stainless-steel vat covered with a lid, but by no means air-tight. Several ordinary steel drums of annatto have been kept in this laboratory for over three years during which time they have lost less than 10% of their strength, although they have been opened from time to time for sampling, and although some of them were only half full from the beginning. Samples stored in ordinary glass bottles in the dark have never given as good results as this, although bottles made of a different glass might be better in this respect. The fact that the use of steel drums is standard commercial practice is an indication that iron containers have little or no effect on the stability of annatto under normal conditions of storage, since commercial annatto in some cases may not be used until well over a year after manufacture.

Standardization of commercial cheese colour

The standardization of annatto solutions for use as a cheese colour is a subject of considerable complexity, due to the variation in the form of the colouring matter under different acidity conditions, and to the above-mentioned change in the form of the annatto when adsorbed on the protein in cheese. The *p*H of Cheddar cheese is about 5·0, and it is reasonable to assume that standardization, if carried out on the annatto in solution, should be carried out at this *p*H. Barnicoat records, however, that "attempts to standardize the colour at *p*H 5·5 (as in average Cheddar cheese) have been unsuccessful, as the tintometer readings obtained from such dilute acid solutions of annatto by transmitted light bear no relation to the colours in alkaline solution". The direct corollary of the conclusion in the latter portion of the above quotation appears to be that standardization in alkaline solution (Barnicoat recommended the use of a 1 in 10,000 dilution of annatto in *N*/10 NaOH) will be an unsatisfactory measure of the strength of annatto for use as a cheese colour. Hence it is difficult to understand why he chose the

medium which bears no relation to the conditions in cheese, in preference to the one which resembles them in some respects.

Apart from the question of method of standardization, there appears to the author to be no real basis for the recommendation for the standard strength of commercial annatto solutions, as made by Barnicoat, even under his own system of measurement. In Table VII of his paper, he gives figures for seven samples of commercial annatto which he tested, and in all of these the proportion of red is higher than in his proposed standard, the actual red/yellow ratios being 0·35, 0·16, 0·11, 0·14, 0·20, 0·38 and 0·17, as against 0·10 in the proposed standard. Barnicoat's recommendation of the standard 4·0 yellow, 0·4 red and 0·1 neutral is tantamount to a statement that no sample of commercial annatto which he examined was of the correct shade. The reasons for this contention are not made clear, nor is any evidence brought forward to show that annatto of the shade which he recommends would have any advantage from the point of view of cheese-making over the ordinary commercial product.

Since it appears that neither in alkaline nor in acid aqueous solution is the annatto in the same form as it is in cheese, standardization in solution would only be justifiable if commercial annattos all consisted of solutions of the same single dyestuff or of solutions of two or more dyestuffs which were always present in the same relative proportions. If such were the case, two annattos which gave the same Lovibond readings in solution would dye the casein of cheese to the same extent. The results cited by Barnicoat in Table VII of his paper show that commercial annattos vary not only in concentration, but also in shade (red/yellow). This variation in shade must be due to variations in the nature of the constituents and/or variations in their relative proportions, and it is not safe to assume that the depth of colour in $N/10$ NaOH of each of these constituents runs parallel with its power of dyeing casein. It is quite conceivable that one constituent which had quite a pronounced colour in $N/10$ NaOH might have little or none at the pH of cheese (Barnicoat's statement that there was no correlation between the readings in $N/10$ NaOH and at pH 5·5 seems to support this), or that it might have no power of dyeing casein and might be almost completely removed in the whey. There might also be a constituent in some samples of which the converse was true.

The recommendation for a system of standardization of annatto in solution is not, in the author's view, justifiable unless the system has been checked against the results with cheese itself, using a large number of samples of annatto having different characteristics. If an arbitrary standard shade of cheese were specified, it would only be necessary to determine what proportion of any particular sample of annatto was necessary to produce cheese of this shade, allowance being made for the original colour of the milk. Owing to unavoidable variations in the cheese-making process, a considerable margin of error would have to be allowed, and it would probably be preferable to define the limits within which the shade of the cheese should lie, rather than to

specify one particular shade. Standardization of annatto on this basis would be absolutely equitable, and would depend on no unproved assumptions.

As was mentioned at the beginning of this paper, it would be desirable to lay down a colour standard for each of the three classes of coloured cheese in New Zealand. It would be an economy to use one of these colour standards for the standardization of annatto as well. In this case, it would be desirable to specify the standard colour in such a manner that colour determinations on cheese could be carried out by dairy workers without the necessity for special training or for the purchase of expensive equipment for the purpose. This suggests the use of standard coloured plates with which the cheese could be compared directly. Paper charts have been issued by the Deutschen milchwirtschaftlichen Vereinigung (Hauptvereinigung) for measuring the colour of butter in Germany. These charts are fully described by Mohr & Ahrens (4), while a discussion as to the suitability of various basal media for such charts is given by the same authors (5). The charts each contain four rectangular coloured plates, the first representing butter which is too weakly coloured; the next two the lower and upper limits of correctly coloured butter; and the last, butter which is too highly coloured. The chart is placed directly on the freshly scraped surface of a block of butter, the colour of the butter being observed through circular holes which are cut out of the centre of each plate. The charts are cheap and are not intended for repeated use, so that questions of soiling and of fading on long exposure to light do not arise.

It is suggested that similar charts could be used for the standardization of the colour of cheese. It should not be difficult to obtain a grade of paper having the same texture as a cheese surface. The exact shade required for each class of coloured cheese would have to be decided by mutual agreement among all interested parties. Once decided, the shades should be specified in terms of one of the standard systems of colour measurement, so that they would be reproducible at any time. The form of the charts would have to be somewhat different from that of the charts used for butter in Germany, since only a trier-plug of cheese would be available for measurement under most conditions of cheese grading.

The chart for one of these classes of coloured cheese could also be used as a standard for the testing of annatto cheese colours. If necessary, a further chart could be used to specify the colour of the milk to be used for making the test cheese.

Summary

Standardization of annatto in aqueous solution has been shown to be unsound, and it has been recommended that standardization be carried out on the basis of the depth of colour produced in cheese itself.

A paper colour standard resembling the German butter standard has been suggested for cheese, the same standard being suitable for the evaluation of annatto solutions for cheese-making.

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180. ALCOHOL-GLYCEROL RENNET PREPARATIONS IN CHEESE-MAKING

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The ordinary rennet preparations used in cheese-making are brine extracts usually containing 14–19% salt. Such commercial rennets are never sterile and have a flora which includes micrococci, aerobic spore-formers, moulds and yeasts.

There are three main stages in the bacteriological life of a rennet solution:

(1) The early phase of high bacterial content due to the original vell microflora contamination in manufacture. Many of these organisms are killed by the high salt concentration and the number consequently diminishes.

(2) A long phase of low bacterial content, the organisms not actively proliferating.

(3) A phase of proliferation and deterioration which rapidly increases in intensity. This stage is readily detected by smell and is correlated with marked loss in strength of renneting power. It is reasonable to assume that this latter change is due to the destruction of the enzymes by the proteolytic and haloduric organisms present and occurs sooner or later with all brine rennets.

Bacteriology of rennet

Knowles (1) found rennet to contain 10,000–100,000 organisms per ml. Fresh rennet contained chiefly pigmented cocci and older samples mainly yeasts, moulds and actinomyces. Ritter (2) found that short motile rods occurring in rennet were acetic acid bacteria and that the true *Bact. coli* was usually present in only small numbers. Later (3) he showed that sometimes rennets having high *coli-aerogenes* counts were found and that these were a potential source of danger for cheese-makers. Mattick (4), in an investigation of "stinker" cheese, isolated from the infected cheese a putrefactive organism (*Cl. putrificum* type) which produced an identical foul smell in suitable media. The same organism was also isolated from commercial rennets. Of the materials concerned in making cheese he was able to find the organism only in milk and rennet and concluded that rennet was one possible source of these fault-producing anaerobes.

There is, however, another aspect—that of rennet as a source of desirable types of micro-organisms. Thus as early as 1875 Cohn drew attention to the possible rôle of rennet bacteria in cheese-ripening. Dorner (5) found that the lactic acid bacteria in the rennet flora were not, as previously supposed, chiefly *Str. lactis* and *Tbm. helveticum* but *Str. thermophilus* and *Tbm. lactis*.

Allen & Knowles (6) were unable to find any differences in the flora or

protein degradation of cheese made with whole rennet and with rennet sterilized by filtration. They concluded that the rennet flora does not play any significant part in cheese-ripening. Davis *et al.*(7) found that those types of lactic acid bacteria found in cheese could, with sterile rennet, bring about the same extent of protein degradation in milk as normally occurred in cheese. The effect of whole rennet varied with the vigour of growth of the lactic acid organisms used. An attenuated strain was unable to control the development of the rennet flora with the result that the whole of the milk clot was ultimately digested. This may be regarded as analogous to the sequence of phenomena in a cheese developing a fault such as "stinker".

From a review of the literature it may be concluded that the rennet flora is not essential to the production of good cheese and *may* be the cause of faults.

Experimental

A comparison has been made of alcohol-glycerol and brine extracts of rennet for cheese-making under practical conditions. The experimental results will be considered under the headings: (1) bacteriology, (2) cheese-making aspects, (3) keeping-quality of rennet solutions, and (4) rates of protein breakdown in the cheese.

(1) *Bacteriology*

Whereas brine rennets may have a count as high as 100,000 per ml., the alcohol-glycerol rennet is either sterile or contains only a few organisms per ml. Typical data are given in Table I. It is evident therefore that the use of alcohol-glycerol rennet would practically preclude the possibility of faults in cheese due to the undesirable flora of the rennet.

Table I. *Plate counts of rennet preparations*

No.	Age	Milk agar 30°	Gelatine 22°	Tributyrin agar 30°	Sucrose agar 22°
			Alcohol-glycerol rennets		
1	3 months	—	8 (liq.)	0	9
2	3 weeks	—	—	9 lipolytic	40 *Pen.* 10 pink yeasts
3	22 years	—	31 (non-liq.)	12 lipolytic	50
4	?3 months	0	—	0	0
			Brine rennet		
5	*c.* 14 months	*c.* 80,000 y.	—	—	*c.* 80,000 y.

No.	Age	Yeast dextrose agar 30°	Beer wort agar (*p*H 6·8) 30°	McConkey agar 37°	Milk double-digest agar 30°
			Alcohol-glycerol rennets		
1	3 months	5	0	0	
2	3 weeks	18 *Pen.* 5 pink yeasts	50 *Pen.* 5 pink yeasts	0	
3	22 years	Spreaders	*c.* 150	2	
4	?3 months	0	0	—	0
			Brine rennet		
5	*c.* 14 months	*c.* 80,000 y.	*c.* 80,000 y.	—	*c.* 80,000 y.

y. = yeasts; *Pen.* = *Penicillia.*

(2) *Cheese-making aspects*

A series of cheese were made, with brine rennet and with the alcohol-glycerol rennet. Graders' reports on these cheese are given in Table III. During the cheese-making experiments several observers, not knowing the nature of the experiments, commented on the fine qualities of the curd produced by the alcohol-glycerol rennet. Not only was the curd of good consistency but it also had a "mellow" texture and pleasant aroma. Some observers pronounced the curd to be the best they had ever seen. Several explanations of this have been put forward. Thus the alteration of the enzyme system in rennet by the alcohol and glycerol or the actual physical effect of the glycerol itself on the fat-protein binding in the curd may be regarded as reasonable hypotheses. The results of experiments on this problem will be published later.

(3) *Keeping quality of the rennet preparations*

The alcohol-glycerol rennet preparation loses about 50% of its strength in a year when exposed to light at room temperature. Most brine rennets deteriorate much more rapidly under these conditions (8). It will be evident therefore that, under ordinary conditions, alcohol-glycerol extracts will keep for a very long time.

It has been possible to test a preparation made in 1915. The times of clotting under standard conditions were: (1) fresh stock alcohol-glycerol rennet, 120 sec.; (2) 22-year-old alcohol-glycerol rennet, 138 sec.; (3) Hansen's rennet, 53 sec. The original strength of the 22-year-old alcohol-glycerol rennet is not known but it would be unlikely to be greatly different from that of the fresh stock alcohol-glycerol rennet used.

(4) *Rates of protein breakdown*

Experiments were carried out to determine the relative rates of protein breakdown with brine rennets and alcohol-glycerol extracts. The amounts of enzyme used (brine and alcohol-glycerol) were adjusted to give clotting times as nearly identical as possible. The analytical data of the cheese during the course of ripening are given in Table II. It will be seen that the figures on the whole are remarkably alike. There is a tendency for the alcohol-glycerol rennet cheese to be higher in non-protein nitrogen and in moisture than the brine rennet cheese. It is probable that no importance can be attached to the higher non-protein nitrogen figures of the alcohol-glycerol rennet cheese. The great significance of moisture content near the critical point has already been discussed (9, 10), and the higher non-protein nitrogen figures may reasonably be attributed to the higher moisture content. It may be mentioned that no significant quantitative differences were found in the flora of the two sets of cheese.

Table II

Enzyme	Cheese no.	Age in days	Total moisture	As % of total N: Non-protein N	As % of total N: Total Sörensen N	As % of total N: Non-protein Sörensen N
AGR	58	3	41·16	2·7	0·71	—
Brine	59	3	40·47	2·7	0·85	—
AGR		23	41·38	4·0	1·5	1·3
Brine		23	40·41	6·8	1·5	1·2
AGR	84	4	38·05	4·2	1·1	—
Brine	85	4	37·69	3·5	1·1	—
AGR		22	36·79	8·1	2·4	1·2
Brine		22	36·81	7·5	2·2	1·1
AGR	91	4	36·76	3·8	1·0	—
Brine	90	4	36·47	3·3	1·0	1·4
AGR		27	35·91	9·4	2·4	1·4
Brine		27	35·58	9·7	2·3	1·4
AGR	92	3	35·22	3·7	0·8	—
Brine	93	3	36·41	3·3	0·7	—
AGR		22	35·59	9·6	2·6	1·3
Brine		22	35·67	9·4	2·8	1·3
AGR	95	3	39·59	5·2	1·2	—
Brine	94	3	38·90	4·4	1·0	—
AGR		21	38·67	10·9	3·1	1·4
Brine		21	38·00	9·2	2·6	1·5

AGR = alcohol-glycerol rennet.

Table III. *Graders' reports*

Cheese	Enzyme	Flavour			Body			Texture			Average values: Flavour	Body	Texture
58	AG	8	8	7	9	7·5	8	7	5	5	8	8	6
59	B	6	7	6	8	8	8	8	6	8	6	8	7
84	AG	8	8	7	8	7	9	7	7·5	9	8	8	8
85	B	8	8·5	6	8	9	8	8	7·5	7	8	8	7·5
90	B	7	8	8	9	9	10	9	6·5	9	8	9	8
91	AG	8	8	8	9	10	9	9	7	8	8	9	8
92	AG	7	6	8	9	10	9	9	8	7	7	9	8
93	B	8	7	8	9	8	10	9	8·5	7	8	9	8
94	B	6	9	8	7	9	9	7	7·5	8	8	8	7·5
95	AG	6	0	6	6	8	9	7	6	7	4	8	7

	Summarized averages: Flavour	Body	Texture
Alcohol-glycerol (= AG)	7·0	8·4	7·4
Brine (= B)	7·8	8·4	7·6

All the cheese had exceptionally good body. Being February cheese they were still immature and lacking in Cheddar flavour. All had a clean flavour except 94 and 95, the latter being definitely off. The inferior flavour score of the alcohol-glycerol rennet cheese may be attributed, at least in part, to the higher moisture content of these cheese. This higher retention of moisture would naturally, with experience, be avoided by suitable modifications in the cheese-making process. In these experiments the cheese-maker aimed at identical methods of manufacture. All the cheese were paraffined.

SUMMARY

1. Although chemical analysis, bacteriological analysis and graders' reports failed to distinguish the alcohol-glycerol rennet cheese from the brine rennet cheese, the marked qualities in body and texture during making of the alcohol-glycerol rennet curd, and the sterility or low count of the alcohol-glycerol extract warrant further trials under practical conditions.

2. Where brine rennet may be suspected as the cause of bacterial faults, the alcohol-glycerol rennet could well be used in its place.

3. Alcohol-glycerol rennets have superior keeping-qualities to brine rennets.

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181. INDUCED O/R POTENTIALS, RATES OF GROWTH AND THE VOLATILE ACID PRODUCTION OF LACTIC ACID BACTERIA IN MILK

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Many types of lactic acid bacteria produce a strongly negative potential in milk. The intensity- and capacity-effects and the rapidity of production of this potential (or "reducing condition") are usually related to the rate of growth and acid production in milk, i.e. the rate of fermentation of lactose. Types which, for any reason, fail to produce quickly such a condition of acidity and reduction will usually do so if the milk is supplemented by one or more of the following additions:

(1) a readily fermentable sugar, e.g. dextrose;

(2) an available source of nitrogen, e.g. animal peptone;

(3) "growth factors", e.g. autolysed yeast or beer wort(1,2).

The rate of fermentation of lactose, and the respiratory system of the type appear to be the major factors controlling the fall in potential, and hence the reduction or non-reduction of any O/R indicator in the milk. In the streptococci intense reduction (as indicated by complete reduction or bleaching of litmus) and rapid acid production appear to be correlated directly with the content of flavine (H_2O_2 producing) enzyme and inversely with haemolysin and proteinase production(3).

A point of considerable interest in this respect is whether the various species and different genera (if the latter may be accepted) differ in their reducing intensities under standardized conditions. Such differences, if they exist, have two important aspects:

(1) the control of growth of other types of micro-organisms and of the course of enzymic activity in such processes as cheese ripening, and

(2) their possible usefulness for identification purposes.

The work reported in this paper was designed to explore these aspects.

(1) Different types of cheese have been shown to maintain different and characteristic potential levels(4,5). Further, the evidence that cheese flavour is due to lactobacilli(6) and other micro-organisms(7) is now very considerable. The rH of any system may influence flavour production broadly in two ways:

(*a*) *By inhibiting or promoting the growth of certain types of micro-organisms.* Thus Aubel & Aubertin(8) have shown that while facultative anaerobes can grow over an rH range of 0–20 or greater, obligate anaerobes proliferate only at rH values from 0 to 12. Knight & Fildes(9) have demonstrated that spores of *Cl. tetani* will not germinate in a medium held at an E_h greater than 0·11 V.

at *p*H 7 (*r*H 18). Ingraham(10) found that the germicidal effect of gentian violet is related to the initial E_h of the medium and is due to the poising effect of the oxidized dye, the reduced form not being toxic. Very similar are the findings of Wood *et al.*(11) that O/R indicators from methylene blue upwards (i.e. having a higher E_0) inhibit the growth of *B. megatherium*. It is evident that even aerobes require a certain minimum *r*H for proliferation.

(*b*) *By influencing the velocity of reaction between "labile hydrogen" and the various hydrogen acceptors in biological systems such as milk or cheese.* The end products of fermentation of a heterofermentive lactic acid organism are largely controlled by this phenomenon, e.g. the relative proportions of CO_2, ethyl alcohol, acetic acid, acetoin, etc.

(2) The possible use of final *r*H values as characteristics for identification purposes has not received much attention. However, Gillespie(12) claims that measurements of final potentials are sufficiently constant to permit the differentiation of lactobacilli and spore-forming anaerobes. The reduction of litmus in litmus milk is, of course, a widely used cultural characteristic(13), but the extent of reduction may depend upon the conditions of the test. Thus with certain mastitis streptococci the extent of reduction of litmus varies with the size of the inoculum(14), this phenomenon presumably being due to the presence of "races" in the strain. Other possible factors are age and type of milk, method of sterilizing and shape of culture vessel. Kluyver & Hoogerheide(15,16) have found that the final *r*H of organisms producing a typical alcoholic fermentation is independent of the type of organism and reaches a value of about 9; homofermentive lactic acid bacteria attain a value of 5–6. These characteristic potentials were only obtained, however, in the presence of dyes capable of permeating the cell membrane.

Korr(17) found that the potential is determined by the reaction rates of the dehydrogenase-substrate system (cf. Table II).

The results obtained in the present work may now be considered.

Technique

One batch of fresh, low count, separated milk was divided into five portions and O/R indicators added as follows:

	*r*H
Methylene blue, 1 in 20,000	14·4
Janus green,* 1 in 20,000	12 1st stage 4·5 2nd stage
Litmus, 1% of a 1% solution (1)	6 (?)
Neutral red, 1 in 20,000	3
Safranine, 1 in 50,000	2·5 (?)

* On sterilization Janus green undergoes decomposition to dimethyl safranine and dimethyl aniline.

The media were then filled into tubes $6 \times \frac{5}{8}$ in., plugged with cotton-wool, sterilized by three steamings at 100° C. for $\frac{1}{2}$ hr. and tested for sterility. All tubes were treated in the same way and finally inoculated with an emulsion of a milk culture (0·1 ml. of a 1 in 100 dilution added to 10 ml. of medium),

the inoculum mixed in the medium and the tubes incubated at suitable temperatures (22, 30, 37 or 45° C.).

Records of the change in appearance of the medium were made after 1, 2, 3, 5, 7, 10, 14, etc. days.

The results are summarized in Table I.

The data so obtained indicate the reducing abilities of the various types in pure culture in plain milk. In practice, however, such a condition is exceptional, as lactic acid bacteria usually grow in association with other types which break down the protein, produce "growth factors" and hydrolyse lactose.

In an endeavour to observe the oxidation-reduction behaviour of lactic acid bacteria in milk under the most favourable conditions, a batch of separated milk containing 5% autolysed yeast(1) and 1% dextrose was prepared and treated similarly to the first batch of plain milk, except that the concentration of methylene blue, Janus green and neutral red was 1 in 10,000 and that of safranine 1 in 20,000. Much more rapid growth and consequently more intense reducing conditions were obtained in this medium. The results are also given in Table I which thus summarizes the behaviour of specific types under the least favourable and under very favourable conditions.

It will be observed that there is occasionally less reduction of the dye in yeast dextrose milk than in plain milk. This may be ascribed to the higher concentration of dye and to the fact that clotting frequently takes place much sooner in the reinforced medium.

DISCUSSION OF THE DATA

Results obtained with plain milk

Generally speaking, the intensity of reduction is proportional to the rate of growth in the medium. This is especially noticeable with those types growing poorly or not at all in plain milk. The addition of yeast and dextrose may result in reduction of dyes which are not reduced at all in plain milk. The E_h values induced by growing lactic acid bacteria are thus considerably influenced by the medium. Nevertheless the results indicate that certain types behave characteristically towards certain dyes and that these findings may be of value for identification purposes. It should be noticed that failure to reduce a dye may be due to (1) failure to induce a sufficiently low E_h, or (2) the toxic effect of the dye.

Of the five dyes used, methylene blue is the most toxic, followed by safranine, though the latter was used in a lower concentration. Neutral red is the least toxic and sometimes appears to have a stimulating action.

The most outstanding results are those for *Str. mastitidis* and *Str. thermophilus* with methylene blue, which, at a concentration of 1 in 10,000, is not reduced by these organisms even in the presence of yeast and dextrose. The effect here is probably a toxic one, and it may be observed that both these

Table I. *The reduction of* O/R *indicators by lactic acid bacteria.*

Five main columns are given for the five indicators used in order of E_0 values. The first subcolumn in each main column gives the results in plain milk, the second the results in yeast dextrose milk. The figures give the time in days required to clot the milk and the extent of reduction as follows:

+ + = complete reduction up to less than 1 in. from surface.
+ = partial reduction up to less than 1 in. from surface.
0 = no reduction up to less than 1 in. from surface.

The figures in brackets give reduction values after (or before) clotting where these differ greatly from those at clotting.

Small areas of reduction, complete or partial, at the bottom or in the middle of the tube have been ignored.

The most salient features in the table are printed in heavy type.

	Methylene blue				Janus green				Litmus				Neutral red				Safranine			
	M.		Y.D.M.		M.		Y.D.M.		M.		Y.D.M.		M.		Y.D.M.		M.		Y.D.M.	
Streptococcus I:																				
Str. thermophilus OJ	2	+ +	**>27**	**0**	4	+	1	+ +	1	+	1	+	1	+	1	+	1	(+)	1	+
Str. thermophilus 670	3	+ +	**> 9**	**0**	1	+ +			1	+	2	+	1	+			1	0	2	+
Str. cremoris OJ	3	+ +			3	+ +			3	+ +			3	+ +			3	+		
Str. cremoris 18OJ	1	+ +	2	+ +	1	+ +	1	+	1	+ +	1	+ +	1	+ +	1	0 (+ +)	1	+	1	0 (+)
Str. cremoris BDI	2	+ +	**13**	**0**	2	+ +	2	+	2	+ +	2	+ +			2	+	2	+	2	+
Str. lactis OJ	2	+ +	1	+ +	1	+ +	1	+	2	+ +	1	+ +	1	+ +	1	+ +	1	+	1	+
Str. lactis BDI	2	+ +			3	+ +			3	+ +			3	+ +			3	+		
Str. lactis 662	5	+ +	2	+ +	7	+	2	+	5	+	2	+ +			2	0 (+ +)	4	+	2	+
Str. lactis 2700	3	+ +			3	+ +			5	+ +			4	+ +			3	0		
Str. bovis 2701			2	+ +			2	+			2	+			2	0			2	+
Str. bovis Sh. 1	>50	0			>50	0			>50	0			50	0			38	0		
Str. bovis Sh. 2	>50	0			>50	0											>50	0		
Str. bovis? Z. 1	>50	0 (+ +)			>50	0 (+ +)			>50	0			>50	0 (+ +)			>50	0		
Str. bovis? Sh. 3	>50	0			>50	0							50	0			>50	0 (+)		
Str. inulinaceus OJ	87	0	2	+ +	87	0	2	+	100	0	2	+ +	87	0	2	+ +	87	0	2	+
Str. faecalis OJ	6	+ +	2	+ +	6	+	2	+	5	0	2	+ +	6	+	2	0	6	0	2	+
Str. faecalis 370	11	+ +			7	+ +			11	+ +			11	+ +			11	+		
Str. faecalis 775	11	+ +			7	+ +			11	+ +			11	+ +			11	+		
Str. faecalis 2703	>40	0	2	+ +	>40	0	2	+	40	0	2	+	38	0	2	0	40	0	2	+
Str. faecalis 2707	11	+ +			11	+ +			11	+ +			11	+ +			11	+		
Str. faecalis Z. 1	3	+ +			4	+			3	+			3	+ +			3	+		
Str. glycerinaceus 2702	16	+ +	2	+ +	11	+ +	2	+			2	+ +	16	+ +	2	+	16	+	2	+
Str. liquefaciens 2705	1	+ +			1	+			1	+ +			1 0 (+ +)				1	0 (+)		
Streptococcus II:																				
Str. mycoides OJ	50	0			25	0			6	0			5	+ +			6	0		
Str. mycoides 2704																	21	0	1	+
Str. mycoides 2706													5	+ +	1	+				
Str. mycoides Z. 1																				
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Str. mycoides Z. 99																				
Str. mycoides Z. 100																				

Streptococcus II:																				
Str. pyogenes OJ	50	0			25	0			6	0			5	+ +			6	0		
Str. pyogenes 2400	5	+ +	1	+ +	5	+ +	1	+			2	+ +	5	+ +	1	+	21	0	1	+
Str. pyogenes 325	>40	0 (+ +)	15	0	>40	0 (+)	15	+	>40	0 (+ +)			>40	0 (+)	15	0	>40	0 (+)	15	0
Str. pyogenes McLeod	> 7	0	20	0	5	+ +	20	0	5	0			5	0	20	0	5	+	20	0
Str. pyogenes Z. 1	7	+ +			7	+ +			7	+ +			7	+ +			7	+		
Str. pyogenes Azgas			6	+ +			6	+							6	+			6	+
Str. pyogenes Graves			6	+ +			6	+			6	+ +			6	+			6	+
Str. pyogenes 602			6	+ +			6	+							6	0			6	0
Str. viridans B. 12			6	+ +			6	+							6	+			6	0
Str. mastitidis OJ	**6**	+ +	**>30**	**0**	1	+	1	+	1	0			1	0	1	0	1	0	1	0
Str. mastitidis 3481	**14**	+ +	**>30**	**0**	7	0	1	+	4	0			7	0	1	0	3	0	20	0
Str. mastitidis 3444	**>50**	**0**			6	+ +			3	0			6	+ +			6	0		
Str. mastitidis Z. 62	**5**	+ +			2	+ +			2	0			1	+ +			2	+		
Str. mastitidis Z. 169	**>38**	**0**			7	+ +			3	0			3	0			2	0		
Str. mastitidis Z. 64	**16**	+ +			3	+ +			3	0			3	0			3	0		
Streptococcus III:																				
Leucon. mesenteroides 3274	>90	0 (+ +)	26	+ +	>90	0	5	+	>90	0	26	+	>90	0	8	+	>90	0	8	+
Bc. arabinosaceus 2706	>90	0			>90	0							>90	0			>90	0		
Leucon. mesenteroides X. 5	>90	0 (+ +)	8	+ +	>90	(+)			>90	0	>30	0	>90	0			>90	0	8	+
Leucon. dextranicum X. 22	>90	0 (+ +)	26	+ +	>90	0 (+)	26	0	>90	0	83	+	>90	0	8	+ +	>90	0	26	0
Str. paracitrovorus DN 2	>52	0 (+ +)	8	+ +	>52	0 (+)			>52	0	>30	0	>52	0	8	+ +	>52	0	8	+
Str. paracitrovorus D 22	87	+ (+ +)	13	+ +	87	0	13	+	87	0	>30	0	87	0	13	+	87	0	13	0
Bc. bovis OJ	>90	0 (+ +)			>90	0			>90	0			>90	0 (+ +)			>90	0		
Str. diaceti-lactis 4			2	+ +			2	+			2	+ +			2	+			2	+
Lactobacillus I:																				
Sbm. casei OJ	>52	0			>52	0 (+ +)			>52	0			>52	0			>52	0		
Sbm. casei R 4	11	+ +			7	0			5	0			7	+ +			7	+		
Sbm. casei R 12	5	+ +	18	+ +	5	+ +	5	+	3	+ +	2	+ +	4	0 (+)	5	+	3	+	5	+
Sbm. casei 3253	11	+ +	30	+ +			13	+	4	0	2	0	3	0	2	0	3	+	13	0
L. casei 2–11	7	+ +			7	+ +			7	+ +			7	+			7	+		
L. casei 2–13	5	+ +			7	+ +			7	+ +			7	+			7	+		
L. casei AO 94	3	+ +			5	+ +			3	+ +			3	+ +			3	+		
L. casei-plantarum 2–9	16	+ +			16	+			11	+ +			11	0			11	0		
Sbm. plantarum OJ	87	0			25	0	10	0	19	0	6	+			6	0	15	0	6	+
Sbm. plantarum 3254	7	+ +			7	+ +			6	0			7	+ +			5	0		
Sbm. plantarum 4125	87	0	>30	0	87	0	13	0	87	0	13	+ +	87	+	13	0	87	0	30	0
L. plantarum 1–4	16	+ +			16	0			16	0			16	0			16	0		
L. plantarum 1–8	15	+ +	13	+ +	13	0			15	0	13	+ +	13	0	13	0	13	0	13	+
L. plantarum 2–2					25	0			25	0			25	0			25	0		
L. plantarum AM	13	+ +			13	0			9	0			11	0			11	0		
L. plantarum 11GS1	87	+			87	+			87	0			87	+			87	0		
L. plantarum 12GS1	>86	0	13	+ +	>86	0	13	+	>86	0	13	+ +	>86	0	13	+	>86	0	13	0
L. plantarum 3GS2	>86	0			>86	0 (+)			>86	0			>86	0			>86	0		

Table I (*continued*)

	Methylene blue				Janus green				Litmus				Neutral red				Safranine			
	M.		Y.D.M.		M.		Y.D.M.		M.		Y.D.M.		M.		Y.D.M.		M.		Y.D.M.	
Lactobacillus II:																				
L. thermophilus B5	>25	0	>30		>25	0			7	+	>30	0	13	0	9	+	9	0	30	0
L. thermophilus Pederson	>25	0			>25	0			>15	0			8	+			9	0		
L. thermophilus Whittock	19	0			13	0			>25	0			8	0			19	0		
L. bulgaricus TC	1	+ +	3	+ +	10	(+ +)	1	+	1	+	20	(+ +)	10	(+ +)	2	+	1	0 (+)	3	+
L. bulgaricus 2889	2	+ +			2	+ +			2	0			3	+ +			3	+		
Tbm. Jughurt OJ	2	+ +			2	+ +			2	0			2	0			2	0		
Tbm. helveticum 4113	5	+ +	7	+ +	5	+ +	7	+	4	+ +			5	+ +	7	+	5	+	7	+
Tbm. lactis R8	10	(+ +)	7	+ +	4	+ +	1	+ +	4	+ +			1	+ +	1	+	4	+	1	+
Tbm. lactis R10	2	+ +			1	+ +			2	+ +			2	+ +			2	+		
L. acidophilus 1724	3	+ +	7	+ +	2	+	7	+	4	+ +					7	+	3	+	7	+
L. acidophilus 1899	3	+ +			3	+			4	+ +			3	0			4	+		
L. acidophilus TC	3	+ +			3	+ +			3	+ +			3	+ +			3	+		
L. odontolyticus 1406	21	0	7	+ +	21	0	20	0	21	0	7	+ +	16	0	7	+	21	0	7	0
L. odontolyticus 1407	38	0 (+ +)			>38	0			21	0			21	0			21	0		
Tbm. cereale OJ			3	+ +	>50	0	1	+	>50	0	10	0 (+ +)	>50	0	1	+	>50	0	24	+
L. Delbrücki 4033	>38	0 (+ +)	8	+ +	>38	0 (+)	5	+	>38	0 (+ +)	6	+ +	>38	0	2	0	>38	0	5	+
L. Delbrücki B (lactose fermenting)	3	+ +	1	+ +	3	+ +	1	+	5	+ +			3	+	1	0	3	+	1	+
Lactobacillus III:																				
Bbm. caucasicum OJ	>38	0	>30	0	>38	0	>30	0	>38	0	>30	0	>38	0	30	+ +	>38	0	>30	0
Bbm. longum R1	>52	0 (+ +)	10	+ +	52	0	6	+	52	0	10	+ +	52	0	6	+ +	>52	0	6	+
Bbm. longum R2	>38	0			38	0			38	0			38	0			38	0		
Bbm. breve R3	>52	0 (+ +)	6	+ +	>52	0	19	0			6	+	>52	0	6	+	>52	0	>28	0
L. rudensis	>52	0 (+ +)	30	+ +	>52	0 (+)	8	+	>52	0	>30	0	>52	0	8	+	>52	0	8	+
L. pentoaceticus 947	>52	0	>30	0	>52	0	30	0	>52	0	>30	0	>52	0	35	0	>52	0	30	0
L. pentoaceticus 4037	5	+ +			25	0			8	0			8	0			9	0		
L. brassicae 4036	87	+			87	0			>87	0			87	0			87	0		
L. brassicae L. 28	>87	0 (+ +)	90	+ +	>87	0	90	0	87	0	>90	0	>87	0	90	0	>87	0	25	0
L. brassicae L. 2108	15	+ +			15	+			15	0			15	0			15	0		
L. gracile Ost.	>87	0	26	+ +	>87	0	8	+	>87	0			>87	0	5	+	>87	0	8	+
L. gracile Charlton	>87	0			>87	0			>87	0			>87	0			>87	0		
L. bifidus 2797	>38	0 (+ +)	30	+ +	>38	0 (+)			>38	0	13	+ +	>38	0			>38	0	13	+
L. acidophil-aerogenes 4034	>50	0 (+ +)	15	+ +	>50	0	7	+	>50	0	15	+ +	>50	0	7	+	>50	0	7	0

streptococci grow well in milk, clotting litmus milk in 1–3 days at 37 and 45° C. respectively but without appreciable reduction of the litmus. With methylene blue clotting is greatly delayed or entirely inhibited, the remaining dyes having little or no effect. The poor reducing properties of these two types indicate a fundamental difference in their respiratory-fermentative mechanisms, e.g. the absence of the flavine-enzyme which appears to be associated with powerful reduction and rapid growth. This subject is under investigation. It has recently been suggested that the flavine-enzyme systems may permit the utilization of oxygen (respiration) at low oxygen tensions without toxic effects due to hydrogen peroxide, i.e. hydrogen peroxide may act under these conditions as a hydrogen acceptor, and so fail to accumulate. Such respiration would presumably yield energy for growth purposes, and it has been observed that, with some strains of lactic acid bacteria, the lag phase is shortened under conditions of slight aeration (5). A possible mechanism of the toxic effect of methylene blue is its action as a hydrogen acceptor in the fermentation process. The side tracking of an essential intermediate (such as a reactive triose) in a reaction would thus inhibit the whole fermentation process. If this is the only source of energy of these two organisms the mechanism of the toxic effect would be explained. In any case it would appear likely that, with these types, either the course of fermentation is different or the lactic acid fermentation is the only source of energy. With the flavine-enzyme possessing strains (such as *L. Delbrücki*) methylene blue reduces the yield of H_2O_2 and has a stimulating effect on oxygen consumption, if not on growth (18)

In some cases the reduction or non-reduction of a dye may be used for identification purposes. Thus all strains of *L. plantarum* fail to produce any reduction of safranine in plain milk, whereas all strains of *L. casei* (except one attenuated culture) cause appreciable reduction of this dye. *L. thermophilus*, *L. odontolyticus* and *L. Delbrücki* (*Tbm. cereale*) (non-lactose-fermenting) fail to reduce any of the dyes in plain milk at the time of clotting, while *L. bulgaricus* usually fails to reduce litmus, neutral red and safranine at clotting. The remaining types of Group II, *Tbm. helveticum*, *Tbm. lactis*, *L. acidophilus* and *L. Delbrücki* (lactose-fermenting) reduce all the dyes, including safranine (partially), at clotting. The *bovis-inulinaceus* group of streptococci, Group III streptococci (*Betacoccus*, *Leuconostoc*), Group II lactobacilli (non-milk strains) and Group III lactobacilli also fail to reduce safranine. Safranine-milk is, therefore, of some value for diagnostic purposes.

The *bovis-inulinaceus* streptococci and the Group III streptococci both fail to reduce any of the dyes *and* clot the milk. *Streptococcus III*, however, reduces methylene blue and partially reduces Janus green soon after inoculation without clotting the milk. This phenomenon may be due to the fermentation of dextrose or other fermentable sugars present in small amounts in the sterilized milk. *L. plantarum* usually causes significant reduction of neutral red in plain milk, whereas *Lactobacillus III* only rarely does this. Moreover, *Lactobacillus I* usually causes a complete reduction of litmus in yeast dextrose

litmus milk while *Lactobacillus III* never produces complete reduction, the medium remaining a pale muddy colour.

Results obtained with yeast dextrose milk

The data obtained with the reinforced medium are of less value for diagnostic purposes than those obtained with plain milk. It is noticeable, however, that the toxic effect of methylene blue is still very pronounced for *Str. mastitidis* and *Str. thermophilus*.

Rates of growth and volatile acid production

The ability of lactic acid bacteria to reduce E_h dyes appears to be chiefly dependent on the rate of production of lactic acid. Slow lactic acid formation with the consequently relatively large amounts of by-products results in poor growth and failure to induce low E_h values. Typical data illustrating this point are given in Table II.

Table II

Culture	Total acidity (as lactic acid)	Volatile acidity % of total*	Days to clot milk	Group classification
L. bulgaricus TC	3·15	2·3	3	II
Tbm. lactis R 8	1·68	3·1	2	II
Tbm. helveticum 4113	1·29	3·1	3	II
Tbm. lactis R 10	2·05	3·5	2	II
L. bulgaricus 2889	3·33	3·9	5	II
L. Delbrücki B	1·57	4·5	4	II
Tbm. Jughurt OJ	3·02	4·5	2	II
L. acidophilus TC	1·59	5·1	4	II
L. casei R 4	1·46	5·5	10	I
Tbm. cereale OJ	0·27	5·6	—	II
L. plantarum 2–2	1·42	5·9	22	I
Sbm. casei R 12	1·19	6·2	5	I
L. acidophilus 1899	2·21	6·5	4	II
L. acidophilus 1724	2·33	10·5	4	II
Sbm. plantarum 3254	1·24	11·2	5	I
L. odontolyticus 1406	1·14	11·7	12	II
Sbm. casei 3253	1·76	12·0	5	I
L. casei 2–11	1·22	12·6	5	I
L. casei-plantarum 2–9	1·21	17·3	12	I
L. plantarum 1–8	1·09	20·3	30	I
L. brassicae L. 2108	1·33	20·8	20	III
L. odontolyticus 1407	0·78	22·3	19	II
L. plantarum 1–4	0·91	23·5	22	I
L. pentoaceticus 947	1·04	26·3	>67	III
Bbm. longum R 2	0·95	28·6	>45	III
L. pentoaceticus 4037	0·78	31·0	30	III
Bbm. caucasicum OJ	0·43	33·2	45	III
L. bifidus 2797	0·64	43·6	>19	III
L. brassicae 4036	0·54	57·0	67	III

* These figures have not been corrected for the small amount of lactic acid volatile in steam. Under the conditions of these experiments about 0·5 % of the lactic acid present would be present in the volatile acid. The correction would merely result in accentuating the differences illustrated in the table.

The volatile acid determinations were made on milk cultures that had been incubated for a short time at 37 or 30° C. and then held at room temperature for a long period (12–24 months), conditions similar to those of well

ripened cheese. The volatile acid was assumed to be acetic acid for purposes of calculation of yield and the results expressed in terms of standard NaOH. The rates of clotting given were those holding at the time of inoculation.

Generally speaking, Group II lactobacilli grow most rapidly in milk (clot in about 2 days at 37° C.) and produce least volatile acid (less than 6%). Group I rods vary from typical *L. casei*, clotting milk in about 5 days at 30° C. and producing 6–12% volatile acid, to typical *L. plantarum* requiring 14–30 days or longer to clot milk and producing 17–24% volatile acid. Finally Group III rods require 1–2 months (or even longer) to clot milk and produce from 20 to 50% of volatile acid. It is interesting to note that this general relationship—rate of growth: fall in potential: amount of by-products—holds not only for the main groups and highly differentiated types, e.g. *L. casei* and *Lactobacillus III*, but also in those cases where strains have altered their characteristics on long culture in the laboratory (Table II). Thus *L. plantarum* 3254 clotted milk in about 5 days and produced only 11·2% volatile acid, whereas the other more typical strains 2–9, 1–8 and 1–4 required 12, 30 and 22 days to clot milk and produced 17·3, 20·3 and 23·5% volatile acid respectively.

It would seem therefore that, if the ability to produce by-products is any criterion of flavour production by lactobacilli in cheese and other milk products, the conditions of the medium (e.g. cheese) are as important as the types which may be present. Enforced slowness of growth may, for example, result in the production of flavour substances which, in a more favourable medium, would never be produced. Thus *L. plantarum* under unfavourable conditions may have a metabolism similar to that of *Lactobacillus III*. It is well known in practice that slow ripening is conducive to good flavour.

SUMMARY

The abilities of type cultures of lactic acid bacteria to reduce five O/R indicators in plain milk and yeast dextrose milk have been described and the factors influencing their behaviour in this respect discussed. Methylene blue, litmus and safranine appear to be of some diagnostic value for the differentiation of types. There is a marked correlation between rapidity of growth in milk, ability to reduce O/R dyes and the proportion of by-products. This relationship holds not only for the different groups and types, but also for variations in behaviour observed in species kept under laboratory observation. It is suggested that rate of growth is fundamentally related to the amount of by-products and hence presumably to flavour-producing substances such as diacetyl and acetic acid. Thus in cheese-ripening enforced slowness of growth may be as important as the presence of specific types.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION C. DAIRY CHEMISTRY

THIS review records the progress of dairy chemistry during the period extending from the end of that dealt with in a previous review(1) to August 1937; the subject will be dealt with under the same broad subdivisions as in the previous review.

A new monthly technical journal, *Dairy Industries*, covering the technology of dairy products, including ice cream, appeared in August 1936 (published in London). Two new books by Harvey and Hill, *Milk: Production and Control* (1936) and *Milk Products* (1937) (Lewis, London) have appeared. These books discuss the elementary chemistry of milk and its products in detail but contain few references to original research. The reviewer has published *The Chemistry of Milk* (Chapman and Hall, London, 1936) which deals with the composition of milk, chemistry of the constituents, physical and nutritional chemistry of milk and milk processing. A second edition of *Milk and Milk Products* by Eckles, Combs and Macy (McGraw-Hill Book Co., New York, 1936) has also appeared.

In the progress of dairy chemistry during the last two years, the subjects which have aroused greatest interest are the composition and oxidation of butterfat; the physical chemistry of milk; the minor constituents and the analysis of milk and its products.

I. THE COMPOSITION OF MILK

(*a*) VARIATION IN COMPOSITION

Turner(2) states that the two main factors affecting the composition of milk are the genetic and the environmental factors. The feeding of high-protein rations has little influence on composition. The feeding of vegetable fats may raise, but feeding fish oils tends to lower, the fat content. The feeding of minerals, with the exception of iodine, does not increase the minerals in milk. It has been found generally that when the fat in milk increases, so also do the protein and ash, but the lactose decreases, and vice versa. Uniformity of composition follows the feeding of well-balanced rations, but on a low ration the fat rises and the yields of milk and fat decrease. The effect of drought is to increase fat and protein but to decrease the lactose content. Young animals give milk of a higher fat content than old animals; this is particularly so in the high-fat breeds. Jacobson(3) calculates from the analytical results of 10,000 samples of New England milk that for every 0·10% rise in fat above the average there is a corresponding rise of 0·04% in the solids-not-fat.

Oxley(4) has found a small negative correlation between yield and percentage of fat. The mean morning fat percentage is always lower than the evening, but the total yield of fat is greater in the morning than in the evening owing to the greater yield. The milk and butterfat yields decrease uniformly throughout the lactation period, but the percentage of butterfat shows an initial decrease and then an increase. Csukas(5) has found that the 12 night hours supply 49·0% of the milk and 41·6 of the whole fat of a day's yield with three milkings per day, while with two milkings it supplies 56·4% of the milk and 49·8% of the fat. Hartley & Baker(6) report an unusual case (Nigeria) of the morning milk being higher in fat content than the evening milk. This was the case in wet seasons, the reverse being the case in dry

seasons. This occurrence is not due to yield differences as the dry season yield of evening milk was only slightly lower than that in the wet seasons; this phenomenon is associated with marked climatic differences and cannot be controlled by the ration of the cows. Stuffe(7) has found an increase in fat and ash content on removing cows from low to high altitude.

Dahlberg *et al.*(8), in investigations on the effect of mastitis on milk composition, have found only slight differences regardless of the degree of infection. There are small decreases in lactose, solids-not-fat and specific gravity, but increases in chloride and albumin. They state that milk which is normal in appearance and behaviour is also of normal composition.

Ranges of composition of milk found by some workers are given in the following table:

Fat (average) %				Author	Country
Winter 3·64		Summer 4·16		Susiura(9)	Mongolia and Manchuria
Fat	Lactose	Protein	Ash		
3·5–4·0	5·0	3·3–3·5	0·75	Florentin(10)	Paris district

The cases of adulteration of milk reported annually by the Ministry of Health(11) account for roughly 7·2% of the samples examined by public analysts.

1934–35: 5506 out of 76,930 (7·2%);
1935–36: 5798 out of 78,674 (7·4%);
1936–37: 5706 out of 80,082 (7·1%).

Shah(12) has reported the detailed compositions of the colostrum of three Montgomery (India) cows. Total solids, acidity and ash reach a constant value in 24 hr. while protein reaches a quarter of its original value in 36 hr. after parturition.

(*b*) Milk of other species

Pissarewsky(13) has reviewed extensively the composition and properties of buffalo milk produced in all parts of the world. The milk is valuable for cheese and butter making; the milk does not cream on standing but the fat can be removed by centrifuging. The range of composition is: fat 5·0–17·0 (average 7·8)%; casein 3·2–9·5%. Godden & Puddy(14) have reported the analyses of samples of milk from eight ewes taken over a period of 2 years. The average composition is: total solids 19·3, total protein 6·1, casein 4·6, albumin and globulin 0·9, lactose 4·8, fat 7·4, and ash 0·97%. The calcium content of the ration has no influence on the composition.

Bergman & Turner(15) have examined goat colostrum. The composition was fat 8·2, total solids 18·8, protein 5·7, casein 3·7 and lactose 3·4%. Normal milk was secreted on the 3rd to 4th day.

II. THE CHEMISTRY OF MILK CONSTITUENTS

(*a*) Milk-fat

Composition. Bosworth & Helz(16) report the presence of an optically active monohydroxy palmitic acid of [α] 2·40–2·47° and M.P. 16·5–17·5° C., in butterfat; the isolated samples always contained a trace of unsaturated acid. The acid is reduced by hydriodic acid to palmitic acid. The lead salt of the acid is soluble in ether and the barium soap in benzene. The same workers(17) obtained 750 g. of methyl esters not distilling over at 245° C. (5 mm. Hg), M.P. 62° C. and B.P. 286° C. (15 mm. Hg) from 360 lb. of butter. The acids were almost pure cerotic (hexacosanoic) acid.

Hilditch and his collaborators have continued their investigations on the minor constituents of butterfat. Green & Hilditch(18) have examined the fat for poly-

ethenoid C_{18} acids by fractionation of the "liquid" acids followed by the separation of the more soluble lithium salts from ethyl alcohol. From the polybromides formed, the mixture was calculated to contain 75% of oleic acid, 1·5% of tri- or tetra-ethenoid C_{18} acids and 23·5% of octadecadienoic acid. The permanganate-oxidation of esters of this last acid suggested that it was a geometric isomer of the same acid from vegetable fats. Oxidation with aqueous permanganate gave tetrahydroxy-stearic acid as was given by the acid from seed fats. They maintain that the small amounts of linoleic acid present in butterfat is obtained by assimilation from food fat and not by synthesis. Hilditch & Paul (19) have investigated the lower unsaturated acids and have found none with less than ten carbon atoms, a trace of decenoic, about 1% of tetradecenoic and 4–5% of hexadecenoic acid. The double bond is in the position 9 : 10 for all acids. This suggests that the lower glycerides of butterfat are produced from oleoglycerides and that the lower unsaturated glycerides are intermediate compounds which have escaped complete saturation.

Arising from his investigations on the minor constituents of butterfat, Hilditch (20) has summarized his views on the connexion between the presence of these minor substances and the unique composition of butterfat. He states that the hypothesis that lower saturated fatty glycerides may be produced *in vivo* from preformed oleoglycerides is not in fundamental conflict with existing knowledge of the precursors of milk fat or of the mechanism of oxidation processes possible in a long carbon chain. When cod-liver oil is included in a cow's diet, the production of milk-fat, and the contents of the lower saturated acids, myristic and stearic acids, are reduced, whilst the oleic acid is increased and highly unsaturated C_{20} and C_{22} acids characteristic of cod-liver oil appear in place of higher polyethenoid acids (such as arachidonic acid). These changes are consistent with a temporary interference with the oxidation-reduction enzymes concerned with the elaboration of milk-fat. The omission of cod-liver oil from the diet results in the secretion of normal milk-fat after a few days.

Vivario & Beneden (21) classify the fatty acids of butter-fat into four groups; those of low, medium and high molecular weight (saturated) and the unsaturated; they suggest easy methods of determining these groups. They find that when the acids of medium molecular weight are high, the higher saturated acids are low and that the sum of the medium and high saturated acids varies with the low group divided by the unsaturated group.

Maynard *et al.* (22) have investigated the influence of food fat on the unsaturation of milk-fat. They determined the iodine values of the milk-fat from cows fed on rations containing fat of high and low unsaturation. The minimum time taken for a marked change to occur in the iodine value was 18 hr., all cases showing changes in 24 hr. and the maximum change taking place in 3 to 4 days. The change in iodine value was not so marked in the blood lipids. The close relation between food and milk fat indicates that fat metabolism is either very direct or takes place very rapidly.

Riemenschneider & Ellis (23) have investigated the composition of the fat of goat's milk by submitting the acids of 3·5 kg. of fat to 63 fractionations. They find the same minor constituents such as the C_{10}-C_{16}-enoic acids and arachidonic acid, as are found in cow's milk-fat. No linoleic acid was found. Chollet & Camiss (24) find that the iodine value of goat's milk fat is generally higher than that of cow's, but within the range of variation of the latter. The soluble volatile fatty acids are lower and the insoluble volatile fatty acids are higher for the goat than for the cow and the ratio insoluble volatile acids/soluble volatile acids is suggested as a means of detecting a mixture of cow's and goat's butterfat. The ratio for cow's butterfat is 8–15, and for goat's, 36–43.

Physical properties of butterfat. Gallup (25) has suggested as an index of hardness

of butterfat, the number of grams (of mercury) required to force a plunger of 5 mm. diameter and weighing 50 g. through a disc of butterfat of 6 mm. thickness at 20° C.

Goryaev (26) has observed that the solidifying point of butterfat is influenced by the rate of cooling and has suggested that the cooling from 40 to 25° C. should take a standard time of 20 min. Richardson (27) has investigated the transition point of, or the temperature at which a second phase begins to separate from, molten butter fat for 9 samples. Very slow cooling of the fat is advised here also. Gallup *et al.* (28) have attempted to correlate melting time, or the time required for 25 ml. of fat to warm from 0° C. and melt completely when immersed in a bath at 45° C., with the constants of the fat. The hardness index (see (25)) and the melting-point are proportional, but the iodine value is in inverse relationship, to the melting time. They find that a factor other than unsaturation determines the consistency of butterfat.

Whetham & Hammond (29) have examined the factors influencing the degree of yellow colour of butterfat. Breed, yield and stage of lactation mostly influence the colour. The breeds arranged in order of decreasing colour of fat are: South Devon, Guernsey, Jersey, N. Devon, Friesian, Shorthorn, Ayrshire, Dexter, Red Poll, Kerry. A slight increase in colour follows feeding conditions which tend to increase yield due to a greater intake of colouring matter in proportion to fat produced. The colour is highest in the first few weeks of lactation and falls later but, after 180 days of lactation, the colour rises as the butter yield falls and as a greater proportion of the milk-fat is supplied by the food intake. Butterfat coming from body fat is paler than that from the food.

Fat oxidation. The source and mechanism of development of an oxidized flavour in milk have been extensively investigated. The effect of traces of heavy metals continues to be recognized; the suggestion that lecithin is the source of the taint has been further developed, while the effects of homogenization and high-temperature pasteurization in inhibiting the development of the taint have been discussed.

Dahlberg & Carpenter (30) have found an increase in the copper and iron content of milk after processing and have found that the development of oxidized flavour is accelerated for that reason. Mohr (31) finds that "emery flavour" is intensified by heating milk in pasteurizers of tinned copper or in containers of bronze or iron. Nickel has a less marked effect, and tin, chromium, aluminium and stainless steels, no effect. The source of the taint is in the fat layer and the taint is communicated to cream and butter. The taint is similar but not identical with oiliness (32). Copper added to milk after pasteurization and cooling causes a more pronounced taint than when added before these operations. The tainting factor is volatile in steam. Roland *et al.* (33) have found oxidized flavour to be the predominant off-flavour of milk and that the incidence of the taint is greater in milk of high fat content and low bacterial count, i.e. in milk of premium grades.

The parts played by copper, lecithin and ascorbic acid in the development of the taint have been investigated by various groups of workers. Sharp *et al.* (34) explain the influence of high temperature-pasteurization in retarding the development of an off-flavour and destruction of ascorbic acid by assuming the destruction of enzymes accelerating these reactions and that these enzymes are not destroyed at 62·5° C. The effect of copper is more pronounced in the presence of the enzymes. The addition of 100 mg. per litre of ascorbic acid greatly retards the development of the flavour. Holder pasteurization at 145° F. for 30 min. does not appreciably accelerate the destruction of ascorbic acid provided that copper is not added. The enzymes are only slightly weakened by holder pasteurization but are inactivated by heating for 1–10 min. at 170° F. Brown *et al.* (35) have found considerable variation among individual cows in the susceptibility of milk to oxidized flavour. Dry feeding has been

found to increase and pasture to decrease this tendency. The feeding of 1 quart per day of tomato or lemon juice, or 0·5 g. of ascorbic acid, greatly decreases the tendency to oxidized flavour. Anderson (36) ascribes the development of the flavour to deficiencies in the ration, which can be supplied by carrots or artificially dried lucerne (not sun-dried). A period of 7–9 days elapses before the improvement from feeding a good in place of a poor ration takes place and a longer period elapses when a poor instead of a good ration is fed. It appears that carotene is important as one of the materials responsible for inhibiting the development of the taint.

Thurston *et al.* (37) have found that homogenization, prolonged agitation at low temperature, and freezing and thawing of milk reduce or eliminate its susceptibility to flavour development. This lends support to their conclusion that lecithin is the constituent which is affected. Ross (38) has found that homogenization pressures of 500–1000 lb. per sq. in. are partially effective in preventing the flavour, while higher pressures are more effective. This was true when copper was added to the milk even before homogenizing. The theory is advanced that adsorption on the surface of the fat globule forms an almost solid protective layer of protein.

Doan & Myers (39) have found, in investigations on the effect of sunlight on milk, that the paper milk bottle affords protection against a burnt but not against a tallowy flavour. Blue or green paper bottles or blue or green cellophane on glass retards the development of both flavours. Although an oxidized flavour appears first with clear glass bottles, the final degree obtained is greatest with the paper bottle. Oxidized flavour is more prevalent in high-fat, burnt in low-fat milk, and in whole milk both taints occur together. The source of the burnt flavour is the casein- and albumin-free serum; there is a correlation between the degrees of fading of the lactoflavin and the flavour. No bleaching of the lactoflavin occurs when a tallowy flavour due to catalysis by copper is present.

Webb & Hileman (40) have found that the development of off-flavour in the presence of copper is accompanied by a rise in the oxidation potential and that summer milk is able to resist flavour development even at high potentials. Catalysis by oleinase is different in that a high potential is not involved.

In studying the oxidation of pure butterfat, Stebnitz & Sommer (41) have found that bleaching occurs at the end of the induction period of butterfat oxidation and that there is also an increase in the volatile fatty acids. The tallowy flavour is always preceded by a high peroxide content. They found a considerable variation in the stability to oxidation of the butterfat from individual cows; this stability appeared to vary inversely as the unsaturation of the fat. The fat from cows on grass was less susceptible to oxidation due to protective substances entering milk in greater quantity. There was no relation between carotene content and fat stability. Greenbank (42) found that butterfat, steam-distilled to get rid of free fatty acids, did not give a positive Kreis test after storage *in vacuo* for 3 years. Alcott & Mattill (43) find that this moderate antioxidant effect of commercial preparations is due to their low content of lecithin. It is *cephalin* which accounts for the anti-oxidizing effect, but there is no quantitative relationship between the amount of cephalin and the prolongation of the period of induction; probably the monobasic phosphate radicle of the lipoid is responsible.

(*b*) Lactose and other sugars

When milking is done at regular intervals, the lactose contents of morning and evening milks are the same, but variation is considerable with hourly milking. Simultaneous blood and milk samples show no correlation in sugar content; milk samples taken 1 hr. later than the blood samples show a better correlation (44).

Whitnah & Caulfield (45) have attempted to isolate rare sugars from milk by partial or complete freezing of whey to remove most of the lactose. They report the presence of a sugar resembling glucose, to the extent of 40 p.p.m.

The hydrolysis of the lactose of human and cows' milk by lactose is identical. Human milk serum contains glucose in amounts up to 3% of the total sugar, as well as dextrin-like polysaccharides. The glucose from human milk is more readily fermentable than crystalline glucose (46).

(*c*) Proteins

Casein. Theophilus *et al.* (47) have studied the effect of factors in the manufacture of natural-sour casein on its properties (220 samples). Thoroughness of washing is the most important factor. Acidities above and below 0·64% at coagulation and fat content of the skim milk above 0·16% gave inferior colour and odour. Solubility was decreased by setting temperatures above 40° C. and cooking temperatures above 49° C. Low ash content depended on the accurate use of an acidity of 0·64% at coagulation accompanied by thorough precipitation and washing. Cooking at temperatures above 49° C. gave a tough and difficult curd to wash, of low solubility but high viscosity. Details of steps in the process for manufacturing the best quality of casein are given.

Sharp & McInerney (48) in their investigations on the effect of salts on the solubility of casein and paracasein have found that the *p*H zone of precipitation of paracasein is much wider than that of casein. Sodium fluoride, chloride and iodide widened the alkalinity-solubility zone of casein but restricted that of paracasein except that fluoride plasticized it with precipitation of calcium. Sodium iodide had a greater peptonizing action on paracasein than the chloride. With paracasein in sodium chloride concentrations of 0·1–0·5 *N*, there were three zones of solubility at *p*H 2·5, 6·0 and 9·5 and zones of insolubility at *p*H 1·0, 4·5 and 7·0–9·0. Applying these results to the manufacture of processed cheese, it is deduced that texture is influenced by the method of heating, composition, degree of ripening, the *p*H zone, the removal of calcium from, and the peptization of, paracasein.

Casein from colostrum is soluble in slightly acidified 70% alcohol, and resembles Osborne and Wakeman's albumin as regards solubility, but the composition is that of casein (49). There is no essential or regular change in the heterogeneous composition of casein during the first 3 months of lactation. Perov (50) describes a method of preparing crystalline casein by moistening pure casein with alcohol, peptizing in dilute sodium hydroxide and slowly adding the equivalent amount of hydrochloric acid. An opalescence forms in this dilute solution in 2–3 days and hexagonal plates separate out in 6 days. Kopaczewski (51) has studied the effect of anions and cations in the gelling of casein. Gels containing calcium salts liquefy on standing. The ions Cl, Br, SO_4, PO_4, Mg, Cd, La, Ce accelerate, and Ba, Cu, Zn, Al and Sn retard, gelling.

Pederson (52) has analysed the casein of milk plasma by ultracentrifugal methods. The casein is present as a coarse poly-disperse system. In buffers containing salts of sodium and potassium, the casein is a very complex system, the degree of dispersion of which depends on its concentration. There are at least three proteins in the serum: a protein of low molecular weight, Palmer's lactoglobulin and the protein usually called lactoglobulin.

Lampitt & Bushill (53) have investigated the precipitation of casein from milk by calcium phosphate. Solutions of calcium chloride, disodium phosphate and sodium hydroxide are added until the precipitate is flocculent. The precipitate is then dialysed to get rid of excess of phosphate when pure casein remains in solution.

Martin (54) states that the isoelectric point of casein from human milk is at *p*H 4·2 and that it can be precipitated from buffer solutions in the *p*H range, 3·8–4·2.

The manufacture and properties of artificial wool made from casein (or casein rayon) have been described by Ray(55) and others(56, 57).

Other proteins. The heat denaturation of lactalbumin and globulin has been studied by Fischer(58) and Rowland(59). Fischer has shown that the rate of denaturation of globulin is increased by the presence of small amounts of the previously denaturated protein. The denaturing action is ordinarily exponential. Denaturation may consist of the exposure and the subsequent reaction of the NH_2 and SH groups, previously buried in the molecule, assuming a brush-heap structure. The high temperature coefficient of the reaction is probably due to steric changes.

Rowland reports that albumin and globulin denature rapidly at temperatures above 75° C. and that denaturation is complete in 60, 30, 10–15 and 5–10 min. at 80, 90, 95 and 100° C. respectively. There is no change in the non-protein nitrogen up to 100° C., but continuous heating at 95–100° C. forms small amounts of proteose nitrogen; protein hydrolysis is appreciable on heating at 115–120° C. Maximum coagulation occurs on heating at a *p*H 4·75–4·80 at which prolonged boiling does not cause hydrolysis. The coagulable nitrogen is 70% of the total soluble nitrogen. On precipitating with trichloroacetic acid, variations depending on the concentration of acid indicate the presence of a proteose-peptone fraction. If the coagulated soluble proteins are determined at *p*H 4·7 by coprecipitation with casein, the coagulable nitrogen accounts for 76% of the total soluble nitrogen and this is regarded as the maximum of true albumin and globulin in casein-free serum. The enzyme hydrolysis of lactalbumin has been investigated by Miller & Calvery(60). Pepsin alone yields 22% of the nitrogen in the amino form. Further hydrolysis with trypsin and protaminase increases the value to 32%. Peptic and pancreatic enzymes together give 68% amino nitrogen and cleavage of 94% of the linkages.

The non-protein nitrogen of the milk of various species has been studied. Perkins(61) states that this fraction accounts for 5% of the total nitrogen of milk. Urea accounts for half of this fraction, the uric acid content is variable but is higher in milk from low-protein rations. Bhagrat(62) gives the following distribution of nitrogen in the non-protein nitrogen fraction of ass's and cow's milk respectively; melanin 7·0, 6·0; amide 15·5, 12·6; amino 50·1, 52·0; non-amino 2·8, 1·7; basic 25·8, 28·2; arginine 17·6, 11·5; urea 21·2, 20·3 mg. per l.

Protein distribution. Davies(63), reporting on the protein distribution in 96 morning samples taken over a period of 2 years from a typical herd of Shorthorn cows, gives the following average distribution (as percentages of the total nitrogen); protein 93·9, casein 76·7, albumin plus globulin 17·2, albumin 13·4, globulin 4·6, non-protein nitrogen, 6·1. The casein nitrogen in 148 samples of similar milk sampled during the previous 3 years averaged 76·5% of the total nitrogen. The average total nitrogen contents of milk for the two periods were 0·501 and 0·517%.

(*d*) Mineral matter

Calcium and phosphorus. Olson(64) has found that the calcium content of milk is high at the start and end of lactation and is lowest in 6–8 weeks. Phosphorus drops to a normal level at 6 weeks and then decreases to the end of the lactation. The variation in the calcium and phosphorus contents of the milks of 70 cows has been given by Burr & Witt(65). The following ranges (g. per l.) were found:

CaO 1·20–2·04, P_2O_5 1·68–2·75 ($CaO/P_2O_5 = 1$ to 1·08–1·64).

Food high in calcium had no effect on the milk calcium. Slow coagulation with rennet occurred in milks containing less than 1·3% of CaO and less than 1·7% of P_2O_5.

Pyne(66) and Ling(67) have used titration methods for determining the distribution of phosphorus in milk. Pyne precipitates soluble phosphates by the addition of

calcium chloride. All phosphate is then converted to the soluble form by the addition of oxalate. The inorganic phosphate of rennet whey is about 6% less than that of the dialysate. Ling also used the titration of oxalated milk samples to determine colloidal calcium phosphate; the colloidal inorganic phosphate was taken to be the difference between the total inorganic phosphate of milk and the corrected value for that in the corresponding whey. The amount of dissolving of colloidal phosphate by increasing the acidity of milk was found to be equal to the increase in total phosphate in the whey. The calcium in whey increases at a greater rate since that of caseinate also enters. A linear relationship was found between milk and curd acidities.

Of the milk of various species, that of the water buffalo, although it contains only 0·64% of ash contains the highest percentage of calcium in the ash (28 as against 18% for cow's milk). Of the goat breeds, the Toggenburg gives milk of the highest calcium content(68).

Chloride. Caulfield & Riddell(69) give the following average percentages of chloride in the milks (1006 samples) of various breeds: Ayrshire 0·126, Jersey 0·125, Guernsey 0·124, Holstein 0·139. Individual cows gave milk ranging from 0·07–0·29, but 96% of samples were within the range 0·08–0·20%. Davies(63) gives the average value for Shorthorns (96 samples) as 0·086 and from investigations on the milk of other breeds, the average values given by Caulfield & Riddell appear to be very high. Davies(70), examining the individual milks of Shorthorn and Guernsey cows, found an average Cl % of 0·095 in 1935 (994 samples) and 0·092 in 1936 (1074 samples). Blood & Rowlands(71) have found a range of 0·07–0·12%, and state that chloride content cannot be used to indicate the presence of mastitis. Sharp & Struble(72) find little difference in the chloride content of morning and evening milk or in that of individual quarters of the udder. In cases of infection with mastitis the first and last drawn milk are high in chloride but the middle portion is within the normal range. A wide variation occurs in the chloride content of milk from infected quarters. After falling during the first few days of lactation, the chloride content is almost constant for 60% of the period and then increases. Chloride content rises when the daily yield of Jersey and Guernsey cows drops below 15 lb. and Holsteins below 25 lb.

Potassium. Leulier *et al.*(73) have studied the variations in the potassium content of human milk at various times during the day; the following values were found: early morning, 0·043–0·062; midday, 0·046–0·070; and evening, 0·042–0·060%.

(*e*) Enzymes

Peroxidase. The peroxidase test has been used to differentiate between raw and pasteurized creams and butters from such creams. Waters & Zürn(74) have applied the benzidine test to a magnesium sulphate serum from butter, adding hydrogen peroxide and ether, and observing the colour of the aqueous layer. A blue colour developing in less than 30 min. indicates that the cream has not been heated to 80° C., but a pure white denotes a pasteurized cream. Old farmhouse butter and very rancid or tallowy butters do not respond to the test owing to destruction of the enzyme. Müller(75) has used the guaiacum test for peroxidase in creams with equal success and advises the use of benzidine for samples preserved with formaldehyde or for sour milk.

Masek(76) has found that the age of milk affects the Storch test; milk held at 88° C. gave a negative test but tended to give a positive test after 24 hr.; 10 parts of pasteurized mixed with 3 of raw milk gave a positive test but after 24 hr. reacted negatively. An acidity of 0·33% does not interfere with the test. Jensen & Winther(77) have added varying quantities of separator slime before pasteurization and have found that the recovery of the ability to give a positive reaction is a function of the time and temperature of heating and of the amount of slime added. If the time of

heating is too short to destroy the peroxidase of the leucocytes, the tubercular organisms in the slime have not been destroyed and the value of the test for controlling this form of pasteurization is enhanced. Ritter(78) has found that bacteria entering milk after pasteurization can bleach the indophenol dye. The peroxidase test is not sensitive enough for determining the amount of copper added to milk in processing.

The peroxidase reaction of human milk is more intense during the 3rd to 6th month of lactation(79).

Lipase. Reder(80) uses a sterilized milk-olive oil medium for the detection of lipase and claims to be able to detect 0·6 mg. of a lipase preparation in milk in 60 mm., and 0·05 mg. in 24 hr. Kay *et al.*(81) use a tributyrin medium at *p*H 8·5 and distil off the butyric acid. Samples from individual cows and throughout the lactation period show considerable variations in lipase content; lipase is less stable to heat than phosphatase and there is more lipase in serum than in cream, whereas the reverse is true for phosphatase; no relation exists between lipase and phosphatase contents.

Krukovsky & Sharp(82) have met with difficulties in churning cream from the milk of cows in advanced lactation due to lipase action and the concentration of soaps and fatty acids at the air/plasma surface. Pasteurization immediately after separating eliminates the trouble.

Amylase. Schloemer(83) has found that milk contains amylase of optimum temperature, 37° C., and optimum range of *p*H, 6·3–6·7, but these conditions can be varied by the use of activators and buffers. Amylase is closely associated with the serum proteins of milk and its activity depends on the presence of milk salts, especially chlorides. Dialysis inactivates it, but the activity is recovered by the addition of chlorides, especially that of calcium.

Phosphatase. A new test has been put forward by Kay & Graham(84). The phosphatase acts on diphenylphosphate liberating phenol, which is determined colorimetrically by Folin and Ciocalteu's reagent. Two modifications of the test are suggested: (*a*) a short test allowing the enzyme to react at 47° C. for 10 min. and (*b*) at 37–38° C. for 24 hr. Test (*a*) shows gross errors in pasteurization while test (*b*) is claimed to detect heating for 20 instead of 30 min. at pasteurization temperature heating at 1·5° F. below the proper temperature or the admixture of 0·25 % of raw with the pasteurized milk. The technique of the test has also been described in detail elsewhere(85, 86).

Koppejan(87) has studied the kinetics of the reactions involved in the destruction of phosphatase in heated milk. The inactivation is monomolecular and complete destruction at 60, 61 and 62° C. requires 75, 45 and 20 min. heating respectively. A positive test with only 0·1 % of raw in pasteurized milk is claimed. The enzyme is least sensitive to heat inactivation at *p*H 7. Some salts accelerate the heat inactivation, e.g. NaCl, KCl, KBr, KI and Na_2HPO_4. Sulphates, lactates and lactose have no effect.

Other enzymes. The optimum *p*H of milk dehydrogenase (xanthine oxidase) has been found to be 8·0(88). Acid dyestuffs have no effect on the enzyme but basic dyes generally inhibit its action. The active groups appear to be acidic in both the Schardinger enzyme and xanthine oxidase, thus pointing to the identity of the enzymes.

(*f*) Minor constituents of milk

Heavy metals. Gangl & Liedl(89) describe a micromethod for the separation of lead as $PbSO_4$ from 100 ml. of milk and its determination by the dithizone method. Kasahara *et al.*(90) report the presence of from 0·02 to 0·04 p.p.m. of lead in U.S.A. milk and from 0·01 to 0·59 p.p.m. in Japanese milk; dried milk has been found to contain 0·17–3·26 and condensed, 0·17–4·59 p.p.m.

Only slight seasonal variation in the copper and iron contents of pooled Holstein and Jersey milk have been found by Krauss & Washburn (91) and in Shorthorn milk by Davies (63). Using microchemical methods, Brock & Woolf (92) have determined the copper, zinc and manganese contents of milk and colostrum (cow and human). They report lower copper contents than those given by other workers, e.g. 0·028 p.p.m. in cow's milk and 0·16 in colostrum. Human milk is richer in copper (0·46 p.p.m.). The manganese contents were 0·01, 0·007 and 0·007 p.p.m. respectively and were increased by feeding manganese-rich foods such as sugar-beet. The respective zinc contents were 3·4, 5·8 and 3·5 p.p.m., and were higher at end of lactation but showed great fluctuations. The copper content of milk was lower when cows were on pasture than when indoor-fed.

Ritter (93) has used the peroxidase reaction to test for copper in milk, cream, butter and melted butterfat. Fat processed in copper containers gives a stronger peroxidase reaction than fat free from copper. The processing of dairy products in containers of copper and copper alloys is detrimental to flavour and causes tallowiness and similar oxidized flavours. Iron, nickel and zinc are harmful, but aluminium, its alloys, and stainless steel are without effect.

Carbon dioxide. Jackson (94) has found that the CO_2 content of milk is 26–29 % of the volume or about half of that in blood. The CO_2 in blood and milk corresponds roughly to the sum of the Na and K in them. McDowall (95) describes a method of determining the CO_2 content of milk by aspiration and absorption in baryta.

Ammonia. The ammonia content of milk has been used mostly as a test for bacteriological quality and a criterion of efficient preservation. Polonovski (96) has found an average ammonia content of 0·8, increasing on holding or boiling to 0·6–1·5 p.p.m., all the ammonia being derived from casein. Sweetened condensed milk does not increase in ammonia content on storing at 20° C. for 4 days, and is therefore regarded as a perfectly preserved product.

Kluge (97) has found that fresh milk contains 1·3 p.p.m. of ammonia, a value not affected by stage of lactation but increased in milk from infected udders. Holder pasteurization increases the ammonia content only slightly but the presence of faecal matter gives large increases. The presence of protein-splitters may be detected from the ammonia content. Milk containing over 3·5 p.p.m. of ammonia means unhygienic conditions of production, and above 5·0 p.p.m. the milk is objectionable. Burstein & Frum (98) use a lower limit to detect unsanitary conditions, namely 2·3 p.p.m.

Phospholipins. Holwerda (99) defines the true lipin phosphorus of milk as the difference between the phosphorus contents of the ether extracts of whole and skim milk; the amount in milk is 0·7–0·8 mg. %. A monamino phospholipin of fairly constant composition can be extracted from buttermilk or from the non-fatty phase of butter. A water-soluble compound containing phosphorus and having the properties of an ester has been isolated from dried skim milk; the compound was not a phospholipin. Holm *et al.* (100) have studied the chemical nature and distribution of the phospholipins of milk. The average molecular weight is found to be 776.

Horrall (101) finds that butterfat contains a fairly constant lecithin content after the fourth day of lactation. The lecithin content of colostrum increases until the birth of the calf and then decreases. Factory milk contains on the average 0·24 % of the fat as lecithin; there is more lecithin in the fat of milk from infected quarters. The lecithin content of skim milk is about 14 % of the fat. Raw sweet cream contains about 0·43 % of lecithin in the fat. Pasteurized sweet cream butter contains 0·17 % lecithin in the fat. The fat of the corresponding buttermilks contain 19·7 and 17·9 % lecithin in the fat. The lecithin content of butter from sour cream decreases slowly but that from sweet cream is constant for at least 24 days.

Miscellaneous. Lampitt & Rooke (102) and Reichard (103) have published methods for the determination of the citric acid content of milk by the pentabromacetone method. The former workers have isolated the acid from milk powder. The citric acid content decreases to zero as acidity rises. McHargue (104) reports the normal iodine content of Kentucky milk as 30 p.p.m., rising to 400 on adding iodides to the food of the cow.

Trout & Taylor (105) have investigated the effect of feeding beet tops on the odour and flavour of milk. They find the flavour most prominent in evening milk; it was found possible to decrease the taint by aeration and pasteurization. Davies (106) has investigated the fishy flavour of milk produced by cows fed on betaine-containing beet by-products and reports the presence of small amounts of trimethylamine and its oxide in milk.

III. THE PHYSICAL CHEMISTRY OF MILK

(*a*) Physical properties

Heln & Belle (107) and Kopaczewski (108) agree closely in the values they have found for the *surface tension* of milk. The former have found a range of 46·6–55·3 (most samples 49–52) dynes per cm. for milk, 4–5 hr. old, at 18° C.; the latter reports a fixed value of 53 dynes per cm., with little effect due to dilution or skimming off the fat. The value however, increases on agitation and ageing.

Leviton & Leighton (109) have studied *viscosity* relationships in emulsions containing milk-fat. They have found that the degree of dispersion of fat does not affect the viscosity significantly with double homogenization (2500 and 500 lb. per sq. in.). By varying the fat content from 0–36 %, they were able to increase the viscosity up to five times its original value.

Conditions for *foam formation* in milk have been studied by Sirks (110). Foaming by churning is greater at lower than high temperatures for both skim milk and cream; foaming by the bubbling of air is greater than by churning and behaves similarly with temperature. A smaller amount of "oiling off" of the fat occurs with foaming at low temperatures. Maximum foaming in skim milk occurs at an acidity close to the curdling point, but the amount of foam is much less than with sweet skim milk. Acid whole milk and acid cream give larger volumes of foam than the sweet products. The rate of decrease of foam volume on continued churning was also investigated. This rate was greater with pasteurized than with sweet cream owing to a lower adsorption power of the fat globules. Sharp *et al.* (111) have found that foaming and the removal of the foam sixteen times from skim milk at 5, 10, 32 and 50° C. do not decrease the foaming capacity; protein accumulates in the foam to an extent of 0·12–0·68 %. The relative amounts of the major proteins are not changed by repeated foamings. With whole milk a decrease in foaming properties is not observed until churning occurs. They state that the failure of buttermilk to foam readily is not due to the absence of foam-forming substances but to the presence of foam-breaking substances.

In the *freezing* of milk and cream, the fat distribution between the frozen and liquid phases depends on the speed of creaming relative to that of freezing, and the size and shape of the container. The fat content of the frozen portion increases greatly if creaming ability is reduced by heating or homogenization. By increasing the fat content, the diffusion of milk constituents into the liquid phase decreases until, at 25 % fat, it is nil. The destruction of the fat emulsion in freezing is brought about by the pressure developed by the freezing of the water; the formation of smaller crystals such as would be caused by adding sugar to a freezing milk, lessens "oiling off" (112). Rapidly frozen pasteurized milk is more homogeneous in com-

position than the same milk taking 8 hr. to freeze into a cake (113). Restivo (114) has determined the size distribution of fat globules in human and cow's milk; the number of globules in both milks varies from 1 to 6×10^6 and the diameter, 1–25μ. Globules larger than 10μ account for 27% of the fat in human but only for 9% of the fat of cow's milk. Cream separation removes 99% of globules over 10μ, 94% of 5–10μ, and 32% of globules below 0·5μ. The number of fat globules in milk increases enormously as the fat percentage of milk exceeds 3·5%. Sirks (115) has found that the agreement between the Gerber value for fat in milk and the amount of microscopically visible globules is almost (99%) quantitive. The fat separated in the Gerber method for buttermilk is mostly not visible under the microscope in the buttermilk.

Rimpila & Palmer (116) have studied the compounds adsorbed on the fat-globule membranes. By successive washing and centrifuging of cream they find that the protein and phospholipins are not constant for all cream samples, but are constant for any one sample for the fourth to tenth washings. Emulsions of fat in rennet whey, skim or buttermilk have membranes differing in composition from those of natural cream in the percentage and properties of the proteins and phospholipins. The phosphatase activity of the cream is in the membrane and is not removed by washing. The membrane protein does not come from milk plasma; the protein of the membranes of both natural and artificial cream contains a prosthetic group which makes the percentage of nitrogen low.

Tracy & Ramsey (117) have investigated the factors causing cream to whip poorly. Pasteurization at 77° C. has no greater effect on whipping properties than when done at 62° C. Cream of the desired fat percentage (35–40%) whips better when separated at that fat content, than when standardized from higher fat cream by dilution. The addition of citrate to raw whole milk improves the whipping qualities of the cream. Freezing the milk causes the cream to whip faster but gives less overrun and more drainage; pasteurization of cream separated from frozen milk improves whipping properties.

Lampitt & Bushill (118) have advanced two methods (one a control and the other a refined method) for testing the efficiency of homogenization. After centrifuging the homogenized milk in a cylinder, the milk is clotted with rennet at 37° C. In the control test, the fat content of the lower half of the clot is compared with that of the whole milk. In the refined method, the clot is frozen and the fat in various layers determined by gravimetric methods. They find a correlation between the fat content of the lower layers and the homogenizing pressures used. Tracy (119) states that milk must be homogenized at temperatures not lower than 115° F. and at pressures not less than 2000 lb. per sq. in. to prevent visible creaming. Sedimentation in homogenized milk is due to destabilized protein and cells, and can be avoided by clarifying the milk before or after homogenization. Homogenized milk is whiter and more sensitive to sunlight than raw but less susceptible to "oiling off" with freezing and thawing. High temperatures of homogenization give milk a cooked flavour.

(*b*) Curd tension

Riddell *et al.* (120) have investigated changes in curd tension during the lactation period; curd tension decreases rapidly in the first month and reaches a minimum in 2–3 months. The breed of cow is the most important factor in determining curd tension; low to high curd tension results from the following order of the breeds: Holstein, Ayrshire, Guernsey, Jersey. They also state that considerable fluctuations in the values obtained for curd tension by the Hill test can be obtained by variations in temperature, the interval between the addition of the coagulant and the application of the test, and in the proportion of pepsin and calcium chloride added.

A considerable amount of work has been done on the effect of the composition and treatment of milk on curd tension. Kugelmass (121) correlates the property with the calcium and casein content of milk. The lowest tension is given by a milk containing 0·124% of calcium and 2·5% of casein; the curd tension increases in milks analysing above these values. Concentration of rennet has no effect. Curd tension decreases with dilution of milk and increases with temperature up to 40° C. and then decreases. The addition of acid and alkali alters the value, the maximum tension being at *p*H 6·0, but, on adding sugar or gelatin, the zone is displaced to a *p*H range, 5·5–6·5. For the same *p*H, the curd tension depends on the added acid; an increase in firmness is given by the following order of added acids: HCl, H_3PO_4, acetic, lactic and citric acids.

Both Tracy (119) and Caulfield & Martin (122) find that homogenization of milk reduces curd tension. The value is reduced by amounts which vary directly with the homogenization pressure up to 2000 lb. per sq. in. A maximum reduction of 57% (49–61%) can be obtained, there being no differences between fresh and old milk; rehomogenization does not cause a further reduction.

Palmer & Tarassuk (123) challenge Lundstedt's (124) claim that it is the release of lecithin from the surface of fat globules by churning, so that it is absorbed on the casein network, which causes milk of high to change to one of low curd tension. They state that the low curd tension of sweet buttermilk is due to the release of the constituents of the fat globule membrane into the milk. The agitation of milk at temperatures below churning temperatures does not lower curd tension. Butterfat globules do not absorb substances from skim milk which reduce the curd tension of milk plasma but they do from normal rennet whey and the curd tension is reduced.

Some work on the physical chemistry of fruit juice-milk mixtures has been reported by Charley (125), Davies (126) and Courtney (127). Charley suggests the mixing of 1 part of a pure fruit juice containing 45–55% of sugar with 5 parts of milk to give a non-curdling product of *p*H about 5·0. Davies has observed that the buffer curves of natural and synthetic fruit juices are linear but that the milk-fruit juice curves are logarithmic. The *p*H of a milk-fruit juice mixture can be roughly obtained from the formula $pH = 6{\cdot}6 - \log_{10} n\,(8B + 1{\cdot}2)$, where n is the number of millilitres of juice to be mixed with 50 ml. of milk and B is the buffer index of the juice. This equation does not hold for synthetic juices or natural juices of low acidity. The *p*H range at which visible curdling occurs is 4·9–5·3, the proportion of juices to add to reach this acidity is well above that used in practice. Courtney advises the addition of citrus juices, in quantities not sufficient to cause curdling, to produce soft curd milk.

(*c*) Acidity and acid-base equilibria

Caulfield & Riddell (128) report the following average titratable acidities of freshly drawn milk from the various breeds: Jersey 0·179, Guernsey 0·172, Holstein 0·161, Ayrshire 0·160%. Values for milks of individual cows varied from 0·098 to 0·295%. That of ten samples of colostrum averaged 0·44%. The acidity of milk from a group of 36 cows showed a decline throughout the lactation, this being most pronounced at the beginning and the end. The maximum variation between the acidities of two consecutive milkings was 0·038%.

Vaillant (129), in studying the condition of milk for pasteurization, states that milk of an acidity less than 0·14% comes from unhealthy cows, that milk of an acidity of 0·14–0·18% is normal, and that milk above 0·18% is questionable. Coagulation of milk at 15° C. occurs at 0·63–0·67 and that at 140° F. at 0·45% acidity. If milk coagulates at acidities less than these when acid is added, the pasteurizing quality is questioned since the presence of coagulating enzymes from bacteria may be responsible. Mayorov (130) has found no correlation between titratable acidity, *p*H and the

Morres coagulation in the natural souring of milk. *p*H changes rapidly at first but the titratable acidity increases regularly. The Morres coagulation is very sensitive to changes in milk composition. Schwarz & Fischer (131) recommend the use of sterile milk sera instead of buffer solutions as standards for the colorimetric determination of the *p*H of milk. A serum from 2 parts of milk and 3 of methyl alcohol is used for both the standards and the determination.

Considerable attention has been paid to the electrolytic process for the de-acidification of milk. The process consists of passing milk through a vessel containing a series of electrodes from 5 to 40 mm. apart through which a current of 3–25 mA. per sq. cm. is passed (132). Pien & Baisse (133) have disproved the theory that it is the lactic acid which is destroyed in the process, and concur with the reviewer (previous review (1), p. 86) that the alkali chlorides are electrolysed, the free alkali thus formed neutralizing the acid and the halogen entering into protein combination. The casein of electrolysed milk contains considerable amounts of chlorine and, on electrolysing, chloride solutions in which only a small quantity of protein is present, some hypochlorite is formed. This form of de-acidifying is not practical with curdled milk as there is insufficient chloride present; it has been found that the lowering of acidity is best done from an acidity of 0·2%, and that the chloride content of the milk is the important factor in determining the amount of lowering possible. The use of aluminium electrodes is recommended.

IV. MILK PRODUCTS

(*a*) Butter

(1) *Texture.* Lyons (134) has correlated the firmness of butters from various sources with iodine value and stearic acid content. He found New Zealand and Australian butter to be firmer than Irish and Danish butter, and Danish firmer than Irish, due to the relatively lower unsaturation of fats of the firmer butters. The iodine value of butterfat is high when the cows are on grass and the value increases as the season advances. The fertility of the soil has no effect on the composition of butterfat. Changes in firmness at the beginning and end of the grazing season are not wholly due to composition but to an improved physical condition of the fat caused by better cooling facilities. Coulter & Coombs (135) record the benefit to body and texture of butter obtained by rapid cooling of cream of temperatures below 5° C. and churning at as low a temperature as possible.

Richardson & Abbott (136) have observed defects in the consistency of butter from cows fed lucerne hay owing to the abnormal composition of the fat. The sticky and crumbly body is due to the fact that when the cream is held under the same conditions as normal cream, much of the fat crystallizes before churning, and there is too little liquid fat left to give plasticity, coherence and spreadability to the butter. Rapid cooling and churning at once give a butter of better texture. Horn *et al.* (137) report an improvement in the consistency of butter by feeding silage over both dry feeding or pasturage. Platon *et al.* (138) have found that the feeding of certain green foods such as legumes and sugar beet tops confer a fishy flavour to butter, but that cutting down the feeding and replacing with beet pulp, cereal grain and hay minimize the risk of a taint developing. The variation in iodine value of butterfat according to the green material fed can be gathered from the following values: lucerne alone, 45–47; clover alone, 46; 70% lucerne, 30% cereals, 39; Beet tops (75%), 29; clover (40%), 36.

(2) *Aroma and flavour.* The attention paid to the development of aroma in ripening cream and butter has been considerable; methods for detecting and deter-

mining diacetyl and acetoin in starters, cream and butter have been perfected; the mechanism of diacetyl production has been further investigated. Hammer (139), dealing generally with butter flavour, considers that there are two main sources of flavour; the butterfat itself, and the metabolic products of cream-culture organisms. He directs attention to the formation of both diacetyl and acetoin in ripening cream, that the latter slowly oxidizes in butter to diacetyl, that average butter contains 2–4 p.p.m. of diacetyl and that a high content of diacetyl is accompanied by low keeping qualities of the butter during storage.

The Voges-Proskauer reaction for diacetyl and acetoin has been used as a qualitative test for the amount of flavour-precursors in cream and butter. The rate of development and the depth of the red colour obtained when concentrated sodium hydroxide is added to a small amount of a culture containing creatine varies with the depth of flavour communicated to the butter (140, 141).

Barnicoat (142) has improved and standardized the distillation methods of determining diacetyl and diacetyl plus acetoin (as diacetyl). Diacetyl is steam-distilled in an atmosphere of CO_2, and diacetyl plus acetoin distilled in steam (as diacetyl) after oxidation of acetoin with ferric chloride solution. Diacetyl in both distillates is fixed by hydroxylamine; the nickel compound of the dimethylglyoxime formed is then prepared, extracted with chloroform and determined colorimetrically against solutions containing known amounts of the nickel compound. An improvement would consist of the complete drying of the solution in which the nickel compound is formed since the solution of the compound in chloroform when wetted with water is difficult. A gravimetric micro-method in which the nickel precipitate is weighed has been advanced by Kunze (143).

The biochemical studies associated with aroma production have centred largely around the effects of acidity and oxygen content of cultures on the amount of diacetyl formed and the diacetyl/acetoin ratio. Michaelian & Hammer (144) have studied the oxidation of acetoin to diacetyl in butter cultures. They have found that, on adding acetoin to sterile milk and bubbling gases through, including oxygen, no appreciable amounts of diacetyl are formed; they conclude that it is the activity of the citric acid-fermenting organisms which brings about the oxidation. Oxygen is, however, necessary for improving the yield of diacetyl by the activity of these organisms. Virtanen *et al.* (145) state that oxygen is not only necessary for this oxidation but also for increasing the production of acetoin. Starters which produce little acetoin in thick layers give abundant yields in thin layers. Ritter & Christen (146) have found that the addition of citrates increase the amounts of volatile acids, diacetyl and acetoin formed in butter cultures, the amounts varying with the time of incubation. Both the volatile acids and acetoin can vary appreciably without seriously influencing the butter flavour.

Hammer *et al.* (147) have shown that when diacetyl and acetoin are added to a medium in which lactic acid organisms are actively growing, some reduction to 2·3 butylene glycol occurs, the change occurring at *p*H 3·8–4·0. The same change takes place in milk cultures at high and low acidities. The addition of citric acid to milk cultures increases the rates of formation of both acetoin and the glycol. Neutralization of cultures increases the disappearance of acetoin while hydrogen peroxide, fumarate and ice-cold temperature delay the rate of reduction. This points to the fate of acetoin and diacetyl in dairy products. The rate of reduction to the glycol is greater in neutralized than unneutralized butter. The reduction is also fairly rapid in 12% sodium chloride solution. Virtanen & Tarnanen (148) state that diacetyl is not formed by the interaction of 2 mol. of acetoin to form butylene glycol and diacetyl, but only when oxygen acts as a hydrogen-acceptor. They consider that most of the diacetyl is formed in the preparation of the butter, since cream

is ripened under anaerobic conditions, but is churned with as much air as possible. It is the bacteria in butter which are responsible for loss of aroma.

Barnicoat (149) has found acetoin and diacetyl in New Zealand butter made from slightly ripened cream; most could be accounted for by the starter added. Very small quantities of these compounds in butter stored for 6 months were not appreciably changed, but in butters containing 4 p.p.m. there was much loss, and in butter from starter cream some diacetyl was reduced to acetoin.

The addition of culture distillates for controlling butter flavour has been suggested by Ruehe (150). He suggests adding citrate to 24 hr. old cultures, incubating for a further 24 hr. and distilling off the diacetyl in aqueous solution after adding ferric chloride to the cultures. The diacetyl solution is standardized and added to butter at the time of salting at the rate of 5 p.p.m. Such butter keeps well in storage. Lemoigne & Monguillon (151) support this addition of diacetyl from a natural source as a flavour-improver, but contend that distillates containing 5–7 mg. of diacetyl per l. are not natural products.

Riddet *et al.* (152) have investigated the types of pasture plants responsible for conferring feed flavours on cream and butter. White and red clovers are wholly responsible and luxuriant grasses have no effect. Sweet vernal grass gives a coumarin flavour. The manuring of land improves the grass-growth and indirectly improves the flavour of butter.

Ritter (153) states that fishiness in butter is due to trimethylamine, formed from betaine, or from the choline portion of lecithin by a hydrolytic-oxidizing action catalysed by traces of heavy metals. The flavour can be controlled by the selection of food for the cow, using fresh sweet cream and flash-pasteurizing at 90° C.

(3) *Keeping quality and storage.* The factors influencing the keeping quality of butter have been described by Overman (154). Butter from sweet cream stores better than that from ripened cream, and butter from neutralized cream, after ripening with starter, better than that from neutralized self-ripened cream. The neutralizer used has no effect on keeping quality but over-neutralization gives butter of low score and low-keeping quality. Salted butter from sweet cream scores higher when fresh but loses points more rapidly during storage than unsalted butter. The best butters were obtained by churning sweet whole milk; these were saleable after 2 years' storage. Barnicoat (155) has found that butter packed in boxes lined with tin or aluminium foil-coated parchment stores better at 14 than 32° F.; packed in tin boxes, the amount of deterioration is less. Storage at −5° F. is superior to that at 14° F. Butter should be cold-stored as quickly as possible. Hood (156) has found, under Canadian conditions, that wood taint is decreased by the casein-formaldehyde treatment of the inside of butter boxes. Kieferle & Senss (157) report that the following wrappers for butter can be arranged in the following order of increasing efficiency: unwrapped, parchment, cellophane, ultrament, tin or aluminium foil. Ultrament is a proprietary vegetable parchment impregnated with a yellow vegetable dye.

Gilmour & Arup (158) claim that the acidity of butter as measured by *p*H is the main factor controlling keeping quality. Butters of *p*H below 6·7 cause difficulties in storage. Butters of high flavour and high *p*H keep well, but those of low flavour and high *p*H keep better.

Guthrie *et al.* (159) state that pasteurization at 74° C. destroys all enzymes harmful to butter but that 64° C. for 30 min. does not. Milk enzymes are not inhibited by salt but are by *p*H below 4·7. The numbers of fat and casein splitters in butter are closely correlated with keeping quality. Jacobsen (160) has observed that butters containing the highest amino-nitrogen content have the lowest scores and suggests that a determination of this form of nitrogen should be made to implement tests for flavour and acidity. Knudsen (161) has correlated the quality and catalase

content of 16,000 samples of butter and finds a correlation coefficient (r) of $-0{\cdot}85$ to $-0{\cdot}96\%$.

(4) *Miscellaneous.* Trambics & Pasztor(162) find that butter containing 1·2–2·4 p.p.m. of copper acquires an oily taint in a short time. Commercial butters contain 0–4·5 p.p.m. of iron and 0–2·0 p.p.m. of copper.

French(163) describes a method of preparing clarified butter (ghee) of long keeping quality by ripening cream for a short time (12–18 hr.), thorough washing of the butter and immediate clarification at the correct boiling temperature.

Dirt in butter is determined by treating 100 g. of butter with 200 ml. of 4% borax solution and boiling. The whole is filtered in a Buchner funnel and the filter washed with petrol(164).

(*b*) Cheese

(1) *Rennetting and making.* Powell(165) has confirmed the findings of other workers that pasteurization of milk at 145° F. for 30 min. cooling to 35° C. and rennetting immediately, or ageing at 5, 35 or 65° C. for 3 hr. only slightly retard coagulability; flash-pasteurization at 75° C. has only a slight effect, but longer times of heating at higher temperatures increase the coagulation time.

Koestler and McDowall have continued their researches into the process of cheese-making. Koestler & Petermann(166) have examined the conditions affecting the escape of whey from curd. The causes of coagulation and rate of escape of whey are not directly related; there is no correlation between coagulation time and the volume of whey expelled; separation of whey is faster the more acid the milk. It is the property of the paracaseinate gel to assume a rigid structure which governs the expulsion of whey. Escape of whey is cut down by cooling to 25° C. but is at a maximum at 45° C. A milk difficult to coagulate gives a curd which gives out its whey slowly with much fat entrained, and this milk added to normal milk increases the fat in the whey and delays the time of commencing of escape of the whey but does not decrease the volume of whey. Curd from skim milk does not expel its whey at a greater rate than that from whole milk and the casein content has little effect.

Dolby *et al.*(167), continuing their studies on Cheddar cheese-making, have found that the acidity of whey at running influences the mineral content and the body of cheese. The acidity of cheese is dependent on the acidity of the whey at running and the amount of lactose remaining in the curd. Adding lactose to curd gives a more acid cheese at maturity although there is some unchanged lactose present after 4 months. In whey, there is a close correlation between the *p*H and the concentration of lactic acid under varying conditions of cheese-making; a small effect, however, arises from the variations in the mineral content of whey due to different acidities at running. This is especially so for the whey draining off late in the process. At hooping time, McDowall & Dolby(168) have found that the salt has not penetrated into the interior of the curd particles but that the salt content is uniform in 12 hr. The fermentation of lactose in curd to lactic acid is quantitative. They bring forward evidence that there is bound water in curd to the extent of 0·2–0·3 g. per g. of dry curd, and that a Donnan equilibrium controls the distribution of electrolytes between the curd and water.

Koestler(169) has observed that fresh Emmenthal cheese gives up water to concentrated salt solutions, but that old cheeses do not, and regards this as an index of the power of curd to absorb water. In this cheese the capacity to swell is greatest at the time of hole formation and a variegated cheese is made up of two types of curd with different swelling capacities. The elastic deformation of the cheese is put forward as a physical property depending not on the consistency of the cheese, but on a basic property of the protein.

The relation of curd tension to cheese-making has been studied by Sanders *et al.* (170) who find that the Hill test is not satisfactory for determining the firmness of curd. The rennet test with the Hill curdometer is more satisfactory. The *p*H of the milk bears a relation to coagulation time and curd tension. Homogenization, pasteurization and the presence of mastitis milk decrease rennet curd tension, the last two also increasing the time of coagulation.

A greater uniformity in the quality of cheese from pasteurized milk has been observed (171) but such cheese requires a longer time for ripening.

McDowall (172) has studied the cheese-yielding capacity of milk. He discusses the ratio of "Walker casein"/fat in relation to the yield of cheese per lb. of fat. He also submits formula for estimating the yield of green cheese from 100 lb. of milk, from the fat, Walker casein, A.O.A.C. casein and total protein values. The yield of cheese from the fat (F) and Walker casein (C) values is $2{\cdot}18C + 1{\cdot}17F$ lb. per 100 lb. of milk.

(2) *Cheese ripening.* Sherwood (173) has investigated the parts played by pepsin and rennet in the ripening of Cheddar cheese. Pepsin causes less protein breakdown than rennet: the type of nitrogen distribution observed in the ripened cheese is characteristic of the enzyme used. Increasing the rennet leads to a greater extent of protein breakdown but with no change in the nitrogen distribution. A rough guide to the comparative breakdown that will occur in cheese is given by measuring the degradation brought about in milk by the coagulating enzymes employed.

In cheese, increases in the amounts of subpeptone nitrogen take place probably due to bacterial action. Sherwood claims that experiments with heat-treated preparations of the enzymes in cheese do not furnish reliable evidence of the parts played by these enzymes in cheese ripening.

Grimmer & Lange (174) have found that the caseogluten formed as a product in the ripening of Tilsiter cheese is not of uniform composition but can be separated into three fractions of different properties and composition. The caseogluten content slowly decreases after 10 weeks' ripening. Parisi & Devito (175) have isolated phosphorylated polypeptides, resistant to acid and enzymatic hydrolysis but easily hydrolysed by alkali and phosphatase, from ripening cheese.

(*c*) Other milk products

(1) *Cream.* England & Mecham (176) have found that the whipping properties of cream and the stability of the whipped product depend on the average size of the fat globules. Guernsey ($16{\cdot}3\mu$) is thus superior to Ayrshire ($10{\cdot}6\mu$) cream. The addition of both sodium alginate and gelatin confers stability but decreases the overrun of the whipped product. The layering of bottled coffee cream (20% of fat) is due to low pasteurizing and separating temperatures, high creaming temperatures, excess of soluble calcium salts and agitation (177). The layer is reduced by using high pasteurization and separating but low creaming temperatures. The acidity of coffee and whipping cream (32% fat) should be 0·12–0·14%. When cream is kept in a container under a pressure of 90 lb. per sq. in. with nitrous oxide, driving out the cream by pressure instantly produces a whipped product (178).

Jackson *et al.* (179) have examined the discoloration and corrosion of the tin container for canned cream. The two main defects are: (*a*) purpling of the tin due to the formation of stannous sulphide, and (*b*) pitting of the can, black patches and black specks in the cream. The purple colour is produced by excessive time and temperature of sterilization; the addition of sodium bicarbonate up to 5 g. per gallon tended to reduce the purpling. Black discoloration occurs only when iron has deliberately been exposed to the action of the cream. Loveless (180) has studied the variation of the viscosity of canned cream with heating. The use of a pre-heating

temperature of 140 instead of 110° F. produces an increase of 50% in the viscosity and decreases the tendency for the cream to become grainy.

(2) *Ice-cream.* The use of sodium alginate as a stabilizer has been described (181). Gelatin and alginate give the same whipping times for 100% overrun, but the alginate product melts easier at room temperature. The addition of 0·2% of pectin improves the body of ice-cream but should be used with 0·3% of gelatin (182). Dahle (183) finds that egg lecithin decreases and soya lecithin lengthens whipping time. The whipping time varies with the amount of "oiling off" of the fat and the butterfat readsorbs a new surface from the constituents of the mix to restore whipping ability: egg-yolk is the best reagent for favouring this readsorption.

Lucas (184) discusses the limitations of the use of skim milk powder and butter in the mix. The precautions necessary for whipping successfully consist mainly of the selection of raw materials of good quality, and proper homogenization. Young (185) suggests overcoming the effect of too much lactose in mixes of high solids content by the use of lactase, caseinates, or the centrifuging of lactose from condensed milk after adding sucrose.

(3) *Condensed milk.* Stebnitz & Sommer (186) have examined the effect of salts on age thickening. A stable condensed milk cannot be further stabilized by the addition of citrate, phosphate or calcium acetate, but an unstable milk can be stabilized by citrate added either before fore-warming or to the finished product. The addition of calcium acetate or sodium phosphate (Na_2HPO_4) to a stable milk causes rapid thickening. There is no correlation between the alcohol test and stability to thickening.

(4) *Whey.* Davies (187) reports the analyses of 20 samples of dried whey. An average analysis is: fat 1·2, protein 12·5, ash 7·5, NaCl 2·3, titratable acidity (as lactic acid) 2·4, lactose 69·4, moisture 7·0%. Methods of drying whey to a cake (188) and on rollers after concentrating (189) have been patented. The precipitate separated from whey at 70° C. (*p*H 4·4–4·8) has been dissolved in sufficient ammonium or sodium bicarbonate, carbonate or hydroxide to reach *p*H 6·4–6·7 and evaporated to dryness at once. This process gives a soluble lactalbumin (190).

V. ANALYSIS OF MILK AND MILK PRODUCTS

(1) *Sampling.* Meade & Leckie (191) have investigated the agreement between the fat tests for composite and for periodical fresh milk samples over a period of 6 months. Considerable variations in fat content occur from month to month, and even from day to day in single herds. The periodical fresh samples showed a fat content 0·09% higher than that of composite samples. 60% of composite tested lower, 12% the same, and 27% above the fresh samples. Taussig (192) has found that weekly, fortnightly and monthly tests give equally satisfactory results.

(2) *Acidity.* In titrating milk with alkali, the *p*H of the end-point with phenolphthalein drops from 9·1 to 8·4 as the concentration of the indicator increases from 0·005 to 0·08% (193). The addition of sufficient indicator to reach the higher concentration is necessary in determining the acidity of milk.

McDowall (194) advocates titration with 0·1*N* NaOH to a standard end-point, namely that equivalent to the colour of a few drops of 0·01% rosaniline acetate in an equal volume of milk.

(3) *Fat content.* Standard apparatus and methods for the determination of fat in milk and milk products by the Gerber methods have been described and specified by the British Standards Institution (195). The quality and dimensions of apparatus, specifications of the reagents used and the methods of carrying out the tests on whole and skim milk, cream, cheese and milk powder are described in great detail.

Golding (196) has found that the standard error of the difference between determinations of fat in milk by the Alfa-Laval (Lindström) and the Gerber methods is 0·05 %. Koenig (197) describes an alkaline method for separating the fat of milk, using sodium hydroxide, Rochelle salt, ammonia and a mixture of alcohols. The separated fat is read in a special testing device; results to within 0·1 % of the Gerber value are claimed. Leithe (198) describes refractometric methods, using bromonaphthalene as a solvent, for determining fat in milk and cheese. The determination of milk fat in cereal foods depends on the Reichert-Meissl and the Kirschner numbers of the extracted fat (199).

(4) *Protein.* McDowall & McDowell (200) determine casein, after acid precipitation, by a formol titration. The casein from 20 ml. of milk precipitated at its isoelectric point, is washed and dissolved in 11 ml. of 0·1 *N* NaOH on a boiling water-bath. After neutralizing to phenolphthalein, 4 ml. of formalin are added and the mixture is titrated to the same end-point. The titration (ml. 0·1 *N* alkali) × 1·05 gives the casein content. Leroy (201) determines total protein by a rapid colorimetric method, claimed to be accurate to 0·1 % of protein, by adding to 10 ml. of neutralized milk phenolphthalein, 1 ml. of neutral formaldehyde and 10 ml. of NaOH solution, containing either (A) 0·625, or (B) 0·745 g. per l. Solution (A) neutralizes milk containing exactly 29, and (B) 35 g. of protein per l. The pink colour formed is compared with standards from which the percentage of protein is read off. Turnau (202) suggests the determination of albumin by the difference in refraction between protein-free and casein-free sera. Casein in cereal-milk foods is extracted at 40° C. with dilute caustic soda, followed by washing with 1 % sodium oxalate and determining the protein by formol titration. The titration value × 0·715 gives the percentage of casein (40 g. material, extract to 500 ml., and use 10 ml. for titration) (203).

(5) *Milk from various species.* Polonovski & Martin (204) differentiate human from cow's milk thus: 1 ml. of milk is neutralized to phenolphthalein; 6 drops of methyl orange are added, and the milk is titrated with 0·01 NH_2SO_4 until the colour changes. Human milk requires 2·5 and cow's milk 10 ml. of acid, and the amount of cow's in human milk can be calculated from the mixture law. Modifications are necessary for samples of sour milk. Human milk gives a red-violet colour on heating with ammonia. Goat's milk in cow's milk can be detected by abnormally high amounts of capryllic acid in the fat (205).

(6) *Adulteration, neutralizers, thickeners.* An attempt has been made to correlate the density and viscosity of milk. The viscosity ranges from 1·58 to 2·00. Tables have been prepared showing values for whole and adulterated milk (206). Mohler & Forster (207) have studied the effect of adding water to milk and cream on a large number of physical constants.

Schwarz *et al.* (208), using three methods of detection, have found benzoic acid in 22 out of 135 samples of processed cheese. MacLachlan (209) has found that accurate results are hard to obtain by the Hehner method for formaldehyde if the sulphuric acid contains sulphur dioxide, the addition of $Fe^{\cdot\cdot\cdot}$ is advised; the effect of adding permanganate to the acid is only temporary.

The curd of butter containing borates does not brown readily on driving off the moisture above 100° C. (210). The curd of milk containing banana sap when treated with hydrochloric acid (1–5 of milk) and boiled for 5 min. shows a pink colour (211).

New methods for the detection of neutralized milk have been suggested. Tapernoux (212) has observed that when 30 ml. of ordinary milk are boiled with 0·25 ml. of 30 % calcium chloride solution a clear serum is given, but that the previous addition of sodium and lithium carbonate and bicarbonate gives a cloudy serum. Evidence of excess of sodium ion has been looked for in many cases. Milk is boiled vigorously for 3 min. and 1 drop of 30 % potassium oxalate is added per ml. of milk. On adding phenolphthalein, neutralized milk will give an intense pink

colour(213). Wille(214) determines sodium in milk as sodium zinc uranylacetate and decides whether neutralizer has been added from the amount found. No account is taken of a considerable variation in the sodium content of milk and no attempt has been made to calculate this value from the chloride content of milk, as suggested by Jones & Davies(215) who found that Cl (mg. %) = Na (mg %) × 1·24 + 18·1.

Considerable difficulty has been met in obtaining reliable results for Stokes's (picric acid) test for gelatin in dairy products in which the protein has broken down somewhat. Richardson(216) has found that clearing the mercuric nitrate filtrate with trichloracetic acid solution gives reliable results for cream. Ferguson & Racicot(217) have met difficulties with sour cream and cottage cheese containing rennet when clearing with lead nitrate. 0·5 % was only faintly positive. Tannic acid, on the other hand, gave positive tests when no gelatine was present and failed to distinguish between rennet and gelatin. The Stokes test was found to be the most useful of all tests.

(7) *Freezing-point.* Stubbs(218) has compared the Hortvet and Monier-Williams apparatus and finds that there is a residual heat transference in both which is greater in the Hortvet. The way to obtain the supercooling factor is described. A new modification of the apparatus is described in which a clearance of 2·5 mm. between the two tubes is provided and in which the alcohol is siphoned off from between the tubes at the time of reading. Oestermann(219) reports cases of abnormal freezing-points of milk from two healthy cows in calf. Values of Δ of 0·499–0·505° and 0·493–0·498° have been obtained. Denis-Lester(220) reports an absence of correlation between Δ and the season of the year. He has found a range of Δ for seven herds of 0·528–0·555° (average 0·542°).

Elsdon & Stubbs(221), in calculating the amount of added water in milk from Δ, take into account the total solids content. Instead of the formula: added water $(W) = 100\,(T - T_1)/T$, they suggest $W = (T - T_1)\,(100 - TS)/T$, where T = average Δ, $T_1 = \Delta$ found, and TS = total solids content.

(8) *Miscellaneous.* Milk solids in bread have been determined on the basis of the lactose content(222). Munsey(223) has found that a calculation from the content of citric acid is unreliable; he extracts all the fat and determines the Reichert number and, by allowing 31·5 for milk fat and 1·0 for cereal fat, calculates the amount of milk-fat from the mixture law. In determining the total solids in condensed buttermilk, zinc oxide should be added to prevent loss of lactic acid by volatilization(224). One-tenth of the lactic acid determined by titration should be added to replace the water lost by neutralization.

Grossfeld(225) describes a method of determining the Kirschner number on small quantities of fats. He obtains values of 18·6–22·0 for cow's butterfat; values for human milk-fat range from 0·8 to 6·4. Human milk-fat thus contains very little butyric and caproic acids. Lucentini & Drago(226) have proposed a new index for butterfat, namely, the number of ml. of 0·1 *N* alkali to neutralize the acids soluble in saturated potassium sulphate. Butterfat gives values of 18·6–20·9; suet gives a value of 3 and cacao butter, 5.

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